

The Basic Nuclear Proteins from Dictyostelium discoideum:

Localisation within the Nucleus and Changes during Differentiation

INAUGURAL-DISSERTATION

zur Erlangung der Philosophischen Doktorwürde
vorgelegt der
Philosophischen Fakultät II der Universität Zürich

von

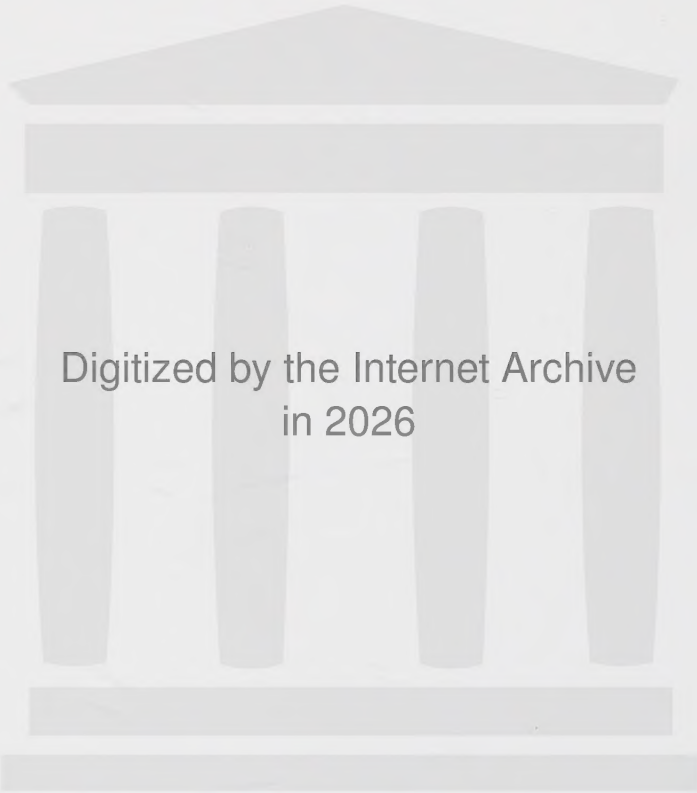
STELIA MARILISA FUHRER-KRÜSI
aus Adelboden (Kt. Bern)

Begutachtet von den Herren
Prof. Dr. R.W. Parish
Prof. Dr. H.R. Hohl

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The Basic Nuclear Protein from Dictyostelium discoideum

Location of the 12 S Basic Nuclear Protein in the Nucleus

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ABBREVIATIONS

BNP	Basic nuclear proteins
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DNP	Desoxyribonucleoproteins
DNA	Desoxyribonucleic acid
HMG	High mobility group
PMSF	Phenyl-methyl-sulfonyl-fluoride
RNS	Ribonucleic acid
SDS	Sodium dodecylsulfate
TLCK	N- α -p-tosyl-lysine chloromethyl-ketone

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I. INTRODUCTION

The mechanism of differentiation in eukaryotes is one of the central problems in biology. Regulation within a cell can occur at various levels. Possibilities are mRNA transport into the cytoplasm, mRNA stability, translational control and the activation/inactivation of enzyme activities. However, cell differentiation is believed to be in the first place a consequence of differential gene activation (1). The chromatin of eukaryotic nuclei consists of DNA, proteins and a small amount of RNA (2), and any one of these alone or in combination could be involved in gene regulation. We are interested in the possibility that nuclear proteins regulate gene expression. This may be a direct control via binding to specific DNA sequences or indirect via chromatin binding leading to local modifications of chromatin structure. The lac-operon in *E. coli* is an example of direct control. Although no gene-repressor or -activator molecules have so far been found in eukaryotic cells there is no reason to exclude such a mechanism. The proteins of nuclei are usually divided into two classes, basic (histones) (3, 4) and acidic (non-histone) (5) proteins.

1. Histones and Chromatin Structure

Histones are a class of heterogeneous (6) basic nuclear proteins, which have been found associated with DNA in the chromatin of higher eukaryotes and in some lower eukaryotes. They are characterized by a high content of lysine and arginine (6). It was originally believed that histones might control gene expression, until it was found that their structure has been remarkably conserved throughout evolution. This is particularly so for the arginine-rich histones (H_3, H_4). Only 2 amino acid substitutions have been found in calf thymus and pea seedling. H_4 histones (7) corresponding to a rate of evolution of 0.06/100 residue/100 million years (8). Histone H_3 from calf thymus, pea seedling and carp testis differ in sequence at only 4 positions (9, 10, 11).

The discovery that histones are highly conserved and show no species or tissue specificity indicated they were unlikely to be specific gene regulators. Their close association with DNA suggested a role in chromatin structure. This idea was subsequently confirmed by the discovery of the nucleosome (for reviews see refs. 12, 13, 14). X-ray diffraction data (15, 16, 17) and electron micrographs (18, 19, 20, 21) showed a regular structure of chromatin in which the DNA double helix was periodically folded by a spherical protein core (22), resembling beads on a string. The nucleosome consists of a variable sized linker region of DNA and a core. Nuclease digestion and cross-linking studies indicate the core particle contains pairs of each of the histones H2A, H2B, H3, H4 (23,24) and 140 base pairs (bp) of DNA (25, 26, 27). Recent work based on cross-linking of histones to DNA (28) demonstrates a chain length of 146 ± 3 bp for the core DNA in higher eukaryotes. X-ray and electron microscopy studies on crystalline nucleosomes indicate that this structure is a flat disc of a dimension of $110 \times 110 \times 57$ Å, and that about 1.75 turns of DNA are wound around the protein core (29). The manner in which DNA and core histones interact in nucleosomes is still unknown, however, many models have been proposed (18, 28-36). The role of histon H1 in chromatin is still controversial.

An attractive current concept suggests that reversible secondary modifications of histones are involved in modulating the chromatin structure. All the histones can be modified enzymatically, either by phosphorylation (37, 38), acetylation (39, 40), methylation (41, 42) or poly(ADP)ribosylation (for review see ref. 43).

The exact biological role of side-chain modification of histones is unknown. However, chemical studies have demonstrated a small number of modified residues, localised in the more basic regions of the histone molecules (44, 45). The ionic interaction between DNA and histones occurs between the positive basic amino acid residues and the negative phosphate groups of DNA. Hence, modification of the basic region of

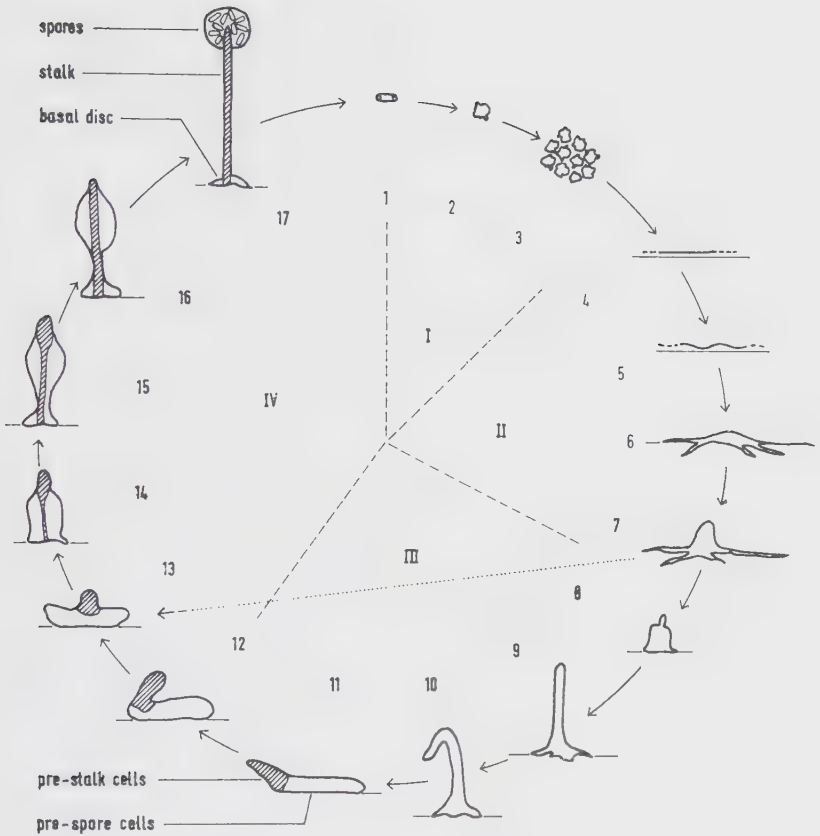


Fig. 1 The Life Cycle of *D. discoideum*

- I Germination and growth: (1) spore, (2-3) myxamoebae
- II Aggregation: (4) very first aggregation, (5) rippling, (6) streaming, (7) aggregate
- III Pseudoplasmodium formation and migration: (8) tip, (9-10) fingers, (11) slug, (12) late slug
- IV Culmination: (13) mexican hat, (14-16) early to late culmination, (17) fruiting body

histones would be expected to change the charge of the proteins and alter their association with DNA. Modification of histones could then bring about subtle changes in chromatin structure capable of influencing gene transcription.

2. Dictyostelium discoideum: a system for studying development

The cellular slime mold *D. discoideum* is an attractive system to study differentiation. Only two cell types differentiate during the life cycle (46) and modulation of gene activity accompanies the morphological changes (47). Development occurs with a high degree of synchrony (48). Further, the *D. discoideum* genome is only 3.6×10^{10} daltons (49, 50) in size. Nuclear DNA represents approximately 76% of the total DNA (51), one third of which is found in reiterated sequences. Although the single copy DNA is theoretically sufficient to code for about 30,000-40,000 genes, recent studies indicate that a large fraction of non-repetitive DNA is not expressed during the life cycle of *D. discoideum* (for review see ref. 52). Experiments using cDNA complementary to polysomal RNA indicate about 3600 genes are required for growth and 400 genes for development (53). Estimations using biochemical (54) and genetic methods (55) provide a lower number : about 500 genes being required for growth, 50-150 for aggregation and 75-140 for later development. The DNA of *D. discoideum* is organized into 7 chromosomes (56). The presence of basic nuclear proteins in nuclei has been demonstrated (57, 58, 59, 60). The presence of nucleosomes has also been confirmed (61, 62).

The life cycle of Dictyostelium discoideum

The life cycle of *D. discoideum* consists of two phases, the growing or vegetative phase and the developmental phase. These two phases have been previously regarded as mutually exclusive stages in the life cycle (63,64). However, Zada-Hames and Ashworth (65) have shown that mitotic cell division of a fraction of the cells may be an integral part

of the developmental phase.

During the vegetative stage the solitary amoebae (myx-amoebae) grow and undergo mitotic cell division with a generation time of 3-12 h depending on the nutrient conditions. On depletion of nutrients the cells pass through a stationary period ("interphase") prior to the developmental phase. During interphase the cells become aggregation competent. Centers are formed to which cells are chemotactically attracted. The chemoattractant is cAMP (66, 67). The subsequent life cycle is summarized in Fig. 1.

3. Aims

The aims of this work were:

- 1) to determine the localization of basic nuclear proteins within the nucleus,
- 2) to examine synthesis of the basic nuclear proteins during differentiation,
- 3) to examine phosphorylation and acetylation of these proteins during differentiation.

II. MATERIALS AND METHODS

1. Cell cultures.

a) Dictyostelium discoideum, axenic strain Ax-3

Cultures in liquid medium: Amoebae were routinely cultured in Fernbach flasks (1,8 l) containing 500 ml of HL-5 medium (68). The flasks were incubated at 23°C in a rotary shaker (55 rpm). Cells were harvested after 44-48 h at a cell density of $5-6 \times 10^7/\text{ml}$.

Cultures on agar plates: Cells cultured in liquid medium were periodically plated ($10^7/\text{ml}$) on 0.1 LP-agar (69) and allowed to differentiate. This ensured a stock of Ax-3 spores, and the capacity of the strain to differentiate could be controlled. Plates were incubated at 23°C in the dark. Fruiting bodies were formed after 2-3 days.

b) Dictyostelium discoideum, wild-type strain NC-4

Cultures in liquid medium: Spores of the haploid NC-4 strain were inoculated in Fernbach flasks containing 500 ml of Sussman's medium (70) with *E. coli* as food source. Cells at the end of the vegetative phase were obtained after a 48 h incubation at 23°C in a rotary shaker (55 rpm).

Cultures on agar plates: Spores of NC-4 were cultured on SM/2-agar (71) with *E. coli* as food source. Fruiting bodies formed after 2 days when the plates were stored at 23°C in the dark.

Differentiation stages of NC-4 were obtained by plating cells, previously grown in liquid medium, onto non-nutrient agar or membrane filters.

Non-nutrient agar was preferentially used when cells were not radioactively labelled. Cells were collected, resuspended in PDF buffer (1/60 M phosphate, 20 mM KCl, 2.6 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 6.5) (72) and plated on buffered 2% (w/v) agar. The plates

were stored at 14⁰ or 23⁰C in the dark, until the requisite stage of differentiation was reached.

Differentiation on cellulose nitrate membrane filters (Schleicher & Schuell) was used for labelling experiments. Collected cells were resuspended in PDF or MES (5 mM morpho-ethanolsulfoacid, 20 mM KCl, 2.6 mM MgCl₂.6H₂O, pH 6.5) (73) at a concentration of approx. 10⁷/ml. Samples of cell suspension (0.5 ml) were plated onto membrane filters overlaying a pad containing 1.5 ml of the corresponding buffer and including streptomycin sulfate (0.5 mg/ml). The cultures were allowed to differentiate at 14⁰ or 23⁰C in the dark.

c) Escherichia coli Br/12

The bacteria were cultured on SM-Agar. Plates were first incubated at 30⁰C for 24 h, and then stored at 10⁰C.

2. Dispersion of aggregates.

Disaggregation experiments and nuclei isolation from various differentiated stages of NC-4 cells required dispersion of aggregates. Cell aggregates were easily dispersed by suspending them in PDF buffer containing 10 mM EDTA (pH 7.5) and stirring slowly for 15 min at room temperature. When necessary the aggregates were gently resuspended with a Pasteur pipette. Cells were then collected and washed once with PDF buffer lacking EDTA.

3. Isolation of nuclei.

Nuclei isolation was achieved by the modified method of Charlesworth and Parish (60). It was necessary to suspend washed vegetative or disaggregated cells of the strain NC-4 in distilled water (0.3 g/ml) and stir slowly for 20 min at room temperature. Cells were then collected by centrifuging at 500 x g for 1-3 min. This first step was omitted when the strain Ax-3 was used, as its plasma membranes seem to be more unstable in distilled water.

Two volumes of cold SF-Medium (0.5 M sorbitol, 2.5% Ficoll,

0.5 mM CaCl_2 , 2 mM MgCl_2 , 50 mM Tris-HCl, pH 7.5) were mixed with 1 volume of distilled water and added to the cell sediment ($4-5 \times 10^8/\text{ml}$, end concentration). An appropriate volume of a 10% (v/v) aqueous solution of Triton X-100 was added to the cell suspension, to give a final concentration of 0.1% Triton, and the suspension stirred for 5-10 min at 18°C . Cell lysis was continually checked with phase-contrast microscopy. All further operations were carried out at $0-4^\circ\text{C}$. The remaining whole cells were removed by centrifuging the cell lysate at $350 \times g$ for 2 min. The supernatant was then centrifuged at $1000 \times g$ for 15-20 min to collect nuclei. The nuclear pellet was sometimes contaminated with large membrane fragments, which interfere with the subsequent purification. The pellet was therefore resuspended in a small volume of SF, and the membrane fragments reduced in size by gently passing the suspension several times through a syringe with an 0.8 mm diameter needle. An appropriate volume of dilute SF (75%) was added to the nuclear suspension to give a final density of about $4-5 \times 10^8$ nuclei/ml. The suspension was layered onto 20 ml 2.2 M sucrose, 0.5 mM CaCl_2 , 2 mM MgCl_2 , and centrifuged at $96,000 \times g$ for 30 min in the SW 27 rotor of a Beckman Spinco L 2 65 B preparative ultracentrifuge. The pellet consisted of highly purified nuclei. Nuclei not used immediately were stored at -20°C .

4. Isolation of chromatin

a) NaCl/water method

This procedure was developed in our laboratories. All operations were carried out at 0°C . Pure nuclei were gently resuspended with a Pasteur pipette for 1 min in 5 volumes 0.15 M NaCl 10 mM Tris-HCl, pH 8.0. The nuclei were pelleted at $3000 \times g$ for 2 min, suspended in 5 volumes distilled water and centrifuged at $3000 \times g$ for 5 min. Washing with distilled water was continued until the pellet was completely free from whole nuclei and appeared as a transparent gel. Nucleoli and colorless spore cases were generally easily separated from chromatin, forming a compact colored

aggregate at the bottom of the chromatin gel. Nuclear membranes remained in the water phase.

When necessary, chromatin was resuspended in distilled water, overlaid on 30% sucrose, and centrifuged 15 min at 1800 x g. The chromatin fraction accumulated at the interphase, nucleoli and spore cases pelleted.

b) Method according to Comings and Harris (74)

Purified nuclei were suspended in 10 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl and stirred 10 min at 4°C. Soluble material was collected by centrifuging at 1500 x g and stored at 4°C, while the pellet was reextracted as above.

Undissolved material was pelleted by centrifuging 15 min at 60,000 x g and the extraction twice repeated. The supernatant was discarded. The pellet consisted of washed but impure "chromatin".

Further purification of the chromatin fraction was attempted by centrifuging the impure chromatin fraction through 1.7 M sucrose at 50,000 x g for 4 h. A pellet was obtained.

5. Isolation of nucleoli

Pure nuclei were suspended in 250 mM sucrose and allowed to swell for 10 min at 0°C. Triton X-100 was then added to a final concentration of 0.5%. The mixture was immediately homogenised in a Sorvall Omnimixer 17220 for 40-50 sec at the maximal power. The suspension was diluted with 2 volumes of cold 10 mM Tris-HCl (pH 7.5) containing 2 mM CaCl_2 . Nucleoli were pelleted through 1 M sucrose at 1300 x g for 15 min at 4°C.

Subsequently, the method was modified to increase the yield of nucleoli. Whole cells were lysed with Triton X-100 as previously described, and the lysate homogenised in a Sorvall Omnimixer for 50 sec at maximal power. The mixture was immediately diluted with 1 volume 20 mM Tris-HCl pH 7.5 containing 4 mM CaCl_2 . Nucleoli were pelleted at 15,000 x g for

15 min, resuspended in 10 mM Tris/2 mM CaCl_2 and centrifuged through a sucrose step gradient (2.2 M/2.0 M/1.8 M/1.5 M) at 100,000 x g for 1 h. The nucleoli remained at the interface of 1.8/2.0 M sucrose.

6. Isolation of DNP-Particles with micrococcal nuclease (75)

Freshly isolated whole nuclei, chromatin or nucleoli were resuspended in 20 mM Tris-HCl pH 7.8. Nuclei were routinely incubated for 5-10 min with 150 units micrococcal nuclease (Worthington; 15,000 U/mg) per ml suspension. Chromatin and nucleoli, which are more sensitive to nucleases, were digested with 75 units enzyme/ml suspension for 2-10 min at 37°C. The reaction was stopped with one tenth volume 0.2 M EDTA (pH 7.5) and cooled at 0°C. Digested material was dialyzed overnight (4°C) against 10 mM Tris-HCl pH 7.5/0.1 mM EDTA/0.1 mM TLCK ($\text{N-}\alpha$ -p-tosyl-L-lysinechloromethyl ketone-HCl, Sigma). Nuclear debris was removed by a 5 min centrifugation at 3000 x g. The preparation was used immediately for characterisation on sucrose gradients or gel-electrophoresis.

7. Isolation of crude nucleosomes

Nuclei or nucleoli were digested with micrococcal nuclease in 20 mM Tris-HCl, pH 7.8, containing 60 mM KCl, 15 mM NaCl, 1 mM CaCl_2 , and dialysed against Tris-HCl/EDTA buffer as described above. After removal of the nuclear debris, the supernatant was made 3 mM with MgCl_2 . The suspension was left 2 h at 0°C (or overnight at -15°C). DNA histone complexes (crude nucleosomes) were pelleted by centrifuging 10 min at 10,000 x g. The proteins remaining in the supernatant were precipitated with trichloroacetic acid (25% final concentration). Crude nucleosomes were characterised on SDS-polyacrylamide gels.

8. Extraction of proteins

a) Isolation of basic nucleoproteins (BNP)

Routinely, basic nuclear proteins were extracted from pure nuclei according to the procedure of Charlesworth and Parish (59). Briefly, fresh or frozen nuclei were resuspended in 10 volumes 1 M CaCl_2 using a glass homogeniser and stirred overnight at 4°C . This step is very important to achieve a good yield of BNP. The CaCl_2 soluble proteins were separated from insoluble material by a 15 min centrifugation at $60,000 \times g$ at 4°C . The supernatant was kept in ice while the sediment was resuspended in CaCl_2 and centrifuged again. The proteins soluble in CaCl_2 were precipitated with trichloroacetic acid at a final concentration of 25%. Proteins were precipitated for 2 h at -15°C and then pelleted by centrifuging at $60,000 \times g$ for 45 min. Proteins were then resuspended in 0.02 N H_2SO_4 using a glass homogeniser and dialysed 12 h against 0.02 N H_2SO_4 and a further 12 h against 0.04 N H_2SO_4 . The acid insoluble proteins and DNA were sedimented by centrifuging at $3000 \times g$ for 10 min. When necessary BNP were precipitated with 10 volumes acetone and dried in vacuo.

b) Isolation of histones according to Wang et al. (76)

Pure nuclei were stirred gently for 2 h in 50 mM sodium phosphate buffer, pH 7.6, containing 5 M urea. The suspension was centrifuged at $20,000 \times g$ for 30 min. Histones were extracted from the pellet with 5 M urea/2.5 M NaCl / 50 mM sodium succinate buffer, pH 5.0.

The viscous mixture was centrifuged overnight at $110,000 \times g$. DNA and associated non-histone proteins pelleted, while histones remained in the supernatant.

c) Isolation of a perchloric acid soluble protein fraction

The perchloric acid soluble fraction was extracted from BNP according to Charlesworth and Parish (59). Briefly, BNP were stirred for 3 h at 0°C in 0.5 M perchloric acid. The extraction was repeated twice. The perchloric acid soluble material

was made 0.02 N in H_2SO_4 and precipitated overnight at -15° after the addition of 10 volumes of acetone. The pellet was collected and dried in vacuo.

9. Fractionation of proteins by gel electrophoresis

a) Chemicals

Acrylamide, N, N'-methylenebisacrylamide (Bis), urea and sodium dodecylsulfate (SDS) were purchased from Serva, Heidelberg. Ammonium persulfate (APS) and N, N, N, N'-tetramethylethylenediamine (TEMED) were purchased from Bio Rad Laboratories, Richmond, California. Thiourea and proteinase K were purchased from Merck, Darmstadt. Gelamide 250 was purchased from Polysciences Inc., Warrington, PA.

b) Acid-area polyacrylamide system of Panyim and Chalkley (77)

Proteins to be electrophoresed were dissolved in 0.02 N H_2SO_4 containing 4 M urea/0.5% 2-mercaptoethanol. The samples were applied to pre-electrophoresed 15% polyacrylamide/0.1% Bis/2.5 M urea/0.9 N acetic acid, gels, pH 2.8. The gels were run with reverse polarity at 2 mA/tube for 3.5 h at room temperature. Gels were removed from the tubes by forcing water between the gel and the tube wall with a syringe. Gels were routinely fixed and stained with 0.1% amido black/20% ethanol/7% acetic acid and destained with 7% acetic acid.

Staining of the gels with Coomassie Brilliant Blue G-250, according to Reisner et al. (78), was also occasionally used. Gels were stained overnight in 0.04% Coomassie Brilliant Blue G-250 in 3.5% (w/v) PCA. (The solution was prepared by stirring for 1 h at room temperature followed by filtering through a Whatmann No. 1 filter). Destaining of the gels was not necessary.

c) Acid-area polyacrylamide system of Hurley (79)

This procedure is a modification of the Panyim and Chalkley system, and is preferred for slab gels. Gels contained 6.25 M urea, 15% acrylamide, 0.5% Bis, 0.9 N acetic acid. Polymerisation of the gels was catalysed by thiourea (0.088 %) and 30% H_2O_2 (4 $\mu\text{l}/\text{ml}$ gel solution). Gels were preelectrophoresed overnight at 80 volts (for a gel 18 x 14 x 0.075 cm). Protein samples were dissolved in 5% acetic acid/2.5 M urea/1 M 2-mercaptoethanol and incubated 1 h at 37°C. Electrophoresis was performed at 80 volts overnight at room temperature.

After electrophoresis gels were removed from the glass plates and stained for 1-2 h in a 1% (w/v) solution of amido black in 50% methanol/50% distilled water/10% acetic acid. Destaining of the gels required several changes of methanol/water/acetic acid (5:5:1). However, this method gave a high background. Hence, gels were fixed and stained in 0.05% Coomassie Brilliant Blue R-250/50% methanol/5% acetic acid for 1 h with simultaneous heating of the solution at 40-50°C in a water bath. Gels were destained with 10% methanol/10% acetic acid at room temperature. The background was reduced and bands rapidly visualized.

d) SDS-polyacrylamide system of Laemmli (80), modified

Electrophoresis was usually performed in vertical slab gels, 11x14x0.075 cm. The stacking gel containing 125 mM Tris-HCl (pH 6.8), 5% acrylamide, 0.15% Bis, 0.1% SDS was polymerized with 0.06% APS and 0.02% TEMED. The running gel containing 460 mM Tris-HCl (pH 8.8), 13.5 or 15% acrylamide, 0.40 or 0.45 Bis, 0.1% SDS, was polymerized with 0.06% APS and 0.016% TEMED. In order to prevent the gel breaking during the drying procedure (see the end of this section) it was necessary to add 0.1% gelamide-250 to the running-gel solution.

Protein samples were dissolved in SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 3% SDS, 5% 2-mercaptoethanol, 10% glycerol)

and incubated in boiling water for 2-5 min. Electrophoresis was carried out at 210 volts for 3 h 10 min- 4 h 15 min at 15°C, with 0.2 M glycine buffer, pH 8.3, 0.1% SDS as the running buffer.

The gels were stained with a two-step-procedure. They were first soaked 1 h in 0.25% amido black/7% acetic acid and then destained with several changes of 7% acetic acid until the background was clear. In order to improve intensification of some protein-bands, the gels were further stained with 0.025% Coomassie Brilliant Blue G-250/25% isopropanol/10% acetic acid. Destaining as described above.

e) Two-dimensional electrophoresis system of Unger-Ullmann and Modak (81)

Two-dimensional gel electrophoresis was performed with vertical slab gels 18x14x0.075 cm in the first dimension, and 18x14x0.1 cm in the second dimension.

The first dimension consisted of the acid-urea polyacrylamide gel system described above (Hurly). Samples to be separated in the second dimension were loaded at twice the normal concentration. After completion of electrophoresis in the first dimension strips were cut out, equilibrated in distilled water for 5-10 min, and then in 0.2 M glycine buffer, pH 8.3/0.1% SDS for 45 min at room temperature. Marker-strips were placed directly in staining solution. Control-marker-strips were first equilibrated and then stained.

The second dimension gel consisted of a SDS-15% polyacrylamide gel, as described above (Laemmli).

After the running gel had polymerised, its upper surface was overlaid with 1.5 cm of stacking gel, consisting of SDS-5% acrylamide. The equilibrated strip from the first dimension was carefully placed on the top surface of the polymerised stacking gel. The strip was fixed in place with the same

stacking gel as above. Electrophoresis was performed at 230 volts for 6 h or at 70 volts overnight (15°C). Staining of the gels was performed as described for acid-urea polyacrylamide and SDS-polyacrylamide gels.

f) Drying of the gels

After fixing, staining and destaining, slab gels were soaked 5-10 min in 50% methanol, to prevent breaking. Gels were then immediately placed between two Cellophane sheets (Celloclair, Switz.) placed on a polyethylene plate, covered with plastic and dried at 60°C in vacuo. Gels were dried for 5-6 h or overnight, depending on gel thickness.

10. Extraction of DNA

DNA was extracted from whole or nuclease digested nuclei, nucleoli, chromatin, and from the corresponding DNP-particles. In order to improve the yield of DNA, samples were incubated with proteinase K (Boehringer) before DNA-extraction. This preincubation was indispensable for DNA extraction from nucleoli or chromatin. Samples were resuspended in 10 mM Tris-HCl/0.4% SDS, pH 8.5, and incubated with 100 μg proteinase K/ml suspension for 3 h at 37°C .

After preincubation with proteinase K, an equal volume of freshly redistilled phenol saturated with 10 mM Tris-HCl/0.1 mM EDTA, pH 7.8, was added to the samples. The mixture was vortexed and centrifuged (1300 x g, 5 min). The DNA of the aqueous phase was extracted twice, and precipitated overnight at -15°C with 2 volumes ethanol. The DNA was washed once with 70% ethanol/30% 10 mM Tris-HCl, pH 7.8/0.1 mM EDTA. The sediment was pelleted by centrifuging 10 min at 18,000 x g, and stored at -15°C .

11. Size distribution of DNA

DNA samples were resuspended in 100 μl 10 mM Tris-HCl, pH 7.8/1 mM EDTA containing 1 $\mu\text{g}/\text{ml}$ RNAase. After a 10 min incubation

the samples were mixed with 100 μ l 80% glycerine/4% SDS/0.1% bromphenol blue. Size distribution of DNA was determined on acrylamide-agarose slab gels (14x11x0.3 cm) according to Compton et al. (82). Gels contained 2 or 5% acrylamide, 0.5% agarose, 40 mM Tris-HCl, pH 7.8. Electrophoresis was carried out at 100 volts for 2 h 30 min at 15°C, with 40 mM Tris-HCl/20 mM sodium azide/2 mM EDTA, pH 7.8, as running buffer. At the end of the run, gels were stained with ethidium bromide (1 μ g/ml in running buffer) for 30 min at room temperature. Gels were photographed under ultraviolet light (254 nm) with a red filter and an Agfapan 100 Professional 120 film.

12. Characterisation of DNP-particles

DNP-particles were isolated as previously described.

a) Fractionation on sucrose gradients

Linear 0-15% (w/w) sucrose gradients (RNAase free, Serva, Heidelberg) in 10 mM EDTA, pH 7.2 (83) were prepared in cellulose nitrate tubes (1x3.5 or 5/8x4 inch, Beckman) with a Beckman density gradient former. The effect of salt concentration (0, 0.5 M, 1.0 M NaCl) in gradients (84) was also tested. DNP-particles were overlayed on the gradients and centrifuged at 95,000 x g for 24 h at 4°C (SW 27 rotor, Beckman). Gradients fractions were collected from the bottom of the tubes and continuously monitored for absorbance at 254 nm (UV Detector 153, Altex). The collected fractions were pooled and dialysed extensively against 10 mM EDTA, pH 7.0, overnight at 0°C. The pooled fractions were then concentrated using Collodion bags (SM 13200, Sartorius, Germany) in vacuo, to a final volume of 0.3-0.5 ml. One half of this volume was analysed for protein, the other half for DNA concentration.

Proteins of each fraction were separated on 13.5% acrylamide/0.1% SDS gel, as previously described. DNA extraction and analysis of the subunits were performed as previously described.

b) Electrophoretic separation of nucleosomes

Our method (developed by Parish et al.) is a modification of the procedures of Todd and Garrard (85) and Varshavsky et al. (86). Glycerine (final concentration 10%) was added to the suspension of DNP-particles and these were loaded on the first dimension gel (2.5% acrylamide/0.5% agarose). The buffer system contained 40 mM Tris-HCl, 3.2 mM sodium acetate, 0.32 mM EDTA, pH 8.0. Electrophoresis was carried out at 100 volts for 3 h at 15°C. At the end of the run gel strips, each containing an electrophoresed sample, were cut out. One strip (control) was first stained with ethidium bromide to visualize DNA, and photographed under ultraviolet light as previously described. The same strip was then stained in 0.05% Coomassie brilliant blue R 250/10% methanol/5% acetic acid. Protein bands appeared after destaining in 10% methanol/10% acetic acid.

A second and a third strip were equilibrated for 10 min in 1% SDS, 86 mM Tris-HCl, 30 mM Na₂HPO₄, 10 mM EDTA, pH 7.8. The second strip was used to examine proteins associated with nucleosomes. For this reason the strip was laid horizontally on a 8% acrylamide/0.5% agarose/0.1% SDS with 36 mM Tris-HCl/30 mM Na₂HPO₄/10 mM EDTA pH 7.8 as the buffer system. The strip was polymerized in place using a 1% agarose solution. Electrophoresis was carried out overnight at 30 volts. The running buffer was as above. Proteins were stained and destained as described for the control strip.

The third strip was used to examine DNA fragments. The gel consisted of the same system as for proteins (see above) but contained only 4% acrylamide. Electrophoresis were carried out overnight at 27 volts. Gels were first incubated for 15 min in isopropanol (25%)-acetic acid (10%) to maintain gel shape. Then SDS was washed out with methanol (50%, 3 x 30 min) before staining with ethidium bromide.

c) Extraction of DNA and proteins from monomer and dimer nucleosomes

Monomer and dimer were cut from first dimension gel and extracted with 1% SDS, 10 mM Tris-HCl, 2 mM EDTA (pH 7.6). After removing the acrylamide by centrifugation the supernatants were extracted with phenol. DNA was recovered from the aqueous phase by ethanol precipitation, protein from the phenol phase by adding an equal volume of 1 M HCl and twelve volumes of acetone.

13. Electron microscopy

Freshly isolated material (nuclei, nucleoli, chromatin) was fixed in 1.2% glutaraldehyde (in 1/15 M phosphate buffer, pH 6.8, 30 min at room temperature) and postfixed in 2% osmium tetroxide (in buffer, for 1 h). Samples were stained in 2% aqueous uranyl acetate prior to dehydration in a graded series of ethanol, and embedded in Spurr's low viscosity medium (87) (standard mixture). Sectioning was performed on a Reichert OM 2 ultramicrotome with a diamond knife. Sections stained with lead citrate were examined either in a Hitachi HS 8 or HU 11 electron microscope.

14. I n v i v o labelling of nuclear proteins

a) Short-term labelling of the cultures

Cells were labelled at various stages of the cell cycle. Growing cells (strain Ax-3) were labelled with isotopes in liquid medium for various times. Differentiating cells were labelled on membrane filters. Strain NC-4 cells were grown in liquid medium (with E. c o l i) and plated on membrane filters (5 for each experiment) as previously described. After reaching a particular stage of the cell cycle, cells were labelled by adding isotopes via a Hamilton syringe to the top of the membrane filter. Labelling was carried out for 2-3 h. Labelled cells were washed carefully with distilled water and dispersed in PDF/10 mM EDTA. Nuclei were isolated as previously described.

Labelling of proteins with (1-¹⁴C) acetic acid. Growing cells were labelled with 300 μCi (1-¹⁴C) acetic acid, sodium salt (40-60 mCi/mmol, Amersham) per 500 ml liquid medium. Differentiating cells were labelled with 15 μCi (1-¹⁴C) acetic acid per membrane filter. Filter pads contained PDF (72) as buffer solution.

Labelling of proteins with (1-¹⁴C) acetyl-coenzyme-A. Differentiating cells were labelled with 4-5 μCi (1-¹⁴C) acetyl co-enzyme A (50 mCi/mmol, Amersham) per membrane filter. Filter pads contained PDF as buffer.

Labelling of glycoproteins with D-(1-¹⁴C) glucosamine. Differentiating cells were labelled with 15 μCi D-(1-¹⁴C) glucosamine hydrochloride (5-10 mCi/mmol, New England Nuclear) pro membrane filter.

Labelling of proteins with L-(2,3-³H) arginine & (4,5-³H (N)lysine. L-(2,3-³H) arginine monochloride (22.14 Ci/mmol, New England Nuclear) and (4,5-³H (N)lysine monochloride (72.13 Ci/mmol, New England Nuclear) were mixed 1:1 (v/v) and neutralised with 1 N KOH. Differentiating cells on PDF buffer were pulsed with 24 μCi amino acids per membrane filter.

Labelling of proteins with inorganic ³²P. Amoebae to be labelled with inorganic ³²P were grown in liquid medium, centrifuged and washed in MES buffer (73) containing 2.6 mM MgCl_2 and 20 mM KCl. Cells were plated out on membrane filters over filter pads containing MES buffer instead of PDF solution. This was necessary since PDF contains phosphate buffer. Differentiating cells were labelled with 12-20 μCi ³²P_i (13.6 mCi/mg, Amersham) pro membrane filter.

b) Long-term labelling of the cultures

In order to detect protein degradation, long-term labelling of the cells was also performed. Amoebae were grown 48 h in

liquid medium with *E. coli*, as previously described. Amoebae were centrifuged, washed in PDF solution, and plated on membrane filters. Cells were labelled with isotopes 10-15 min after plating out for periods between 1 and 20 h. Labelled cells were dispersed and nuclei from various stages of the cell cycle were isolated as described above.

15. In vitro labelling of nuclear proteins

Cells (strain NC-4) were grown in liquid medium with *E. coli* for 48 h, centrifuged, washed, plated on non-nutrient agar plates and allowed to differentiate as previously described. Nuclei, nucleoli and chromatin were isolated at various stages of the cell cycle and labelled with isotopes in an *in vitro* system.

Labelling of proteins with adenosine 5'-(γ - ^{32}P)triphosphate

Endogenous *in vitro* phosphorylation of the proteins was carried out by incubating 20-30 μl nuclei in 100 μl modified Sampson's kinase buffer (88) (20 mM phosphate, pH 7.2, 10 mM MgSO_4 , 10 mM dithiothreitol) and 5 μM adenosine 5'-(γ - ^{32}P) triphosphate-tetra (triethylammonium) salt (3 mCi/mmol, Amersham) for 5 min at room temperature. The reaction was stopped by adding 150 μl SDS sample buffer for gel electrophoresis and heating 2 min in a water bath.

Effect of histones (H1, H2B, H4, from calf thymus, Boehringer) or polyamine (Spermine-tetrahydrochloride, Serva; Spermidine-trihydrochloride, Fluka) on phosphate incorporation as described by Farron-Furstenthal and Lightholder (89) was tested. Effects of 0.14 M NaCl and /or cAMP (10^{-5} - 10^{-7}M) (90) and of cGMP (10^{-6}M) (91) on protein phosphorylation were also tested.

Preincubation assays with nucleases (88) were performed under the following conditions: Digestion of nuclei with 150 U micrococcal nuclease (Worthington, 15,000 U/mg) per ml buffer

(20 mM Tris-HCl, pH 7.8, 60 mM KCl, 15 mM NaCl, 1 mM CaCl_2) for 5 min at 25°C. Reaction terminated with 0.2 M EDTA. Digestion of the nuclei with 300 U DNAase I (Sigma) per ml buffer (100 mM acetic acid, pH 5.5, 5 mM MgSO_4) for 5 min at 25°C. Stopping of the reaction by cooling (0°C) and changing the acidity of the suspension to pH 7.8. Digestion of the nuclei with 300 U DNAase II (Worthington) per ml buffer (100 mM acetic acid, pH 5.5, 1 mM MgSO_4) for 5 min at 25°C. Stopping of the reaction by cooling (0°C) and raising the pH of the suspension to 7.8. One volume of concentrated (2x) kinase buffer was added to the digested nuclei. Incubation with (γ - ^{32}P) ATP was performed as described above, in the presence or absence of cAMP and cGMP (10^{-6} M).

Phosphatase treatment of the phosphorylated BNP (nuclei were labelled prior histone extraction) was performed as described by Sherod et al. (92). Briefly, histones (in 20 mM Tris-HCl, pH 8.2) were incubated with alkaline phosphatase from E. Coli (Boehringer) at 37°C at a concentration of 30 $\mu\text{g/ml}$. The same quantity of enzyme was added after 24 h, and incubation was continued for a further 14 h. Treatment was terminated by adding acetic acid (1/20 volume) and solid urea (500 mg/ml).BNP were precipitated with 10 volumes of acetone.

16. Detection of incorporated radioactivity on gels

Detection of ^3H or ^{14}C labelled protein bands on gels was achieved using the fluorography method of Bonner and Laskey (93). Stained or unstained gels were first soaked in 20 volumes Me_2SO (dimethylsulfoxide) for 30 min., followed by a second incubation in fresh Me_2SO . Gels were then transferred in 4 volumes of 20% (w/w) PPO (2,5 diphenyloxazole) in Me_2SO and soaked for 3 h. They were then washed in water for 1 h. Gels were dried under vacuum and then placed in contact with X-Ray films (Fuji Film) between two glass plates. The X-Ray films were first activated with a flash exposure using a

green filter. The film-gel sandwich was exposed at -70°C for a week or more.

^{32}P labelled gels were stained, dried and directly exposed with non-activated X-Ray films.

III. RESULTS

A. LOCALISATION OF BASIC NUCLEAR PROTEINS WITHIN THE NUCLEUS

1. Isolation of nuclei

The haploid strains Ax-3 and NC-4 (wild type) of *D. D i s - c o i d e u m* were used. The axenic strain Ax-3 was chosen for preliminary experiments and whenever a large amount of cells were required. It was found advisable to collect the cells 44-48 h after inoculation, at a maximal density of $5-6 \times 10^7$ cells per ml, before a red pigment appeared in the medium. Older cells were more resistant to Triton X-100 and isolation of nuclei was difficult. The NC-4 strain (non-axenic) was used for differentiation experiments but had the disadvantage that only relatively small quantities of cells could be obtained.

The use of new culture isolates of Ax-3 and NC-4 cells necessitated a modification of the method previously described by Charlesworth and Parish (60) for the isolation of nuclei. SF-medium and Triton X-100 were retained, and rapid cells lysis of washed cells was obtained at an optimal temperature of 17°C. Cell lysis was usually complete after 5 min, with the release of a large number of nuclei which appeared intact under the phase contrast microscope. Triton X-100, however, did not solubilize a residue of large membrane fragments. Purification by centrifuging through 1.0M sorbitol or a step gradient of 1.0-3.0-4.0 M sorbitol (in 0.05 M Tris, pH 7.5) was unsatisfactory because membrane fragments sedimented with nuclei. Moreover, a large number of nuclei were broken. Centrifugation through 0.5 M sucrose or sucrose step gradients (80-65-50% w/v) containing 0.008% spermidine phosphate in 0.05 M Tris-HCl, pH 7.5 (59), also failed to reduce membrane contamination and nuclear lysis.

These difficulties were finally overcome by disrupting the large membrane fragments by repeated gentle passage through a syringe. The nuclei were then centrifuged through a 2.2 M

sucrose cushion, containing 0.5 mM calcium chloride-2.0 mM magnesium chloride, at 96,000 x g for 30-45 min. Nuclei, stabilised by the presence of divalent cations, were pelleted while membrane fragments were not. Two bands were visible in the sucrose cushion: the lower consisted of aggregates of nuclei and membranes, the upper of membranes and cytoplasmic particles. The pellet consisted of pure nuclei, as shown in Fig. 2. The yield of pure nuclei was about 50-60%.

Clumping and shrinking of nuclei occurred during centrifugation. A short incubation of 5-10 min in SF medium diluted with distilled water (2:1) sufficed to disperse and swell nuclei to the original size.

Isolation of nuclei from NC-4 cells involved two additional problems. Some contamination by *E. coli* was unavoidable when nuclei were isolated from growing or stationary cells, since bacteria released from food vacuoles co-sedimented with nuclei after lysis of the cells. It was also difficult to isolate a reasonable yield of pure nuclei from differentiated stages. Nuclei size actually decreases as differentiation advances. The very small nuclei from the stages between slug and culmination were unable to pass the 2.2 M sucrose cushion. This difficulty was overcome by a 7% dilution of the sucrose solution, but the pelleted nuclei were slightly contaminated with membranous material. A second centrifugation through sucrose was sometimes necessary.

Electrophoretic patterns of the proteins present in nuclei isolated from Ax-3 and NC-4 are compared in Fig. 3. On SDS-PAGE (15%) the pattern was essentially the same, with the two exceptions indicated by arrows. These two bands will not be further considered, as they do not belong to the region (indicated by the numbers 1-14) where BNP are found. It cannot be excluded, however, that dimers, aggregates or modifications of BNP are present in the regions above band 1.

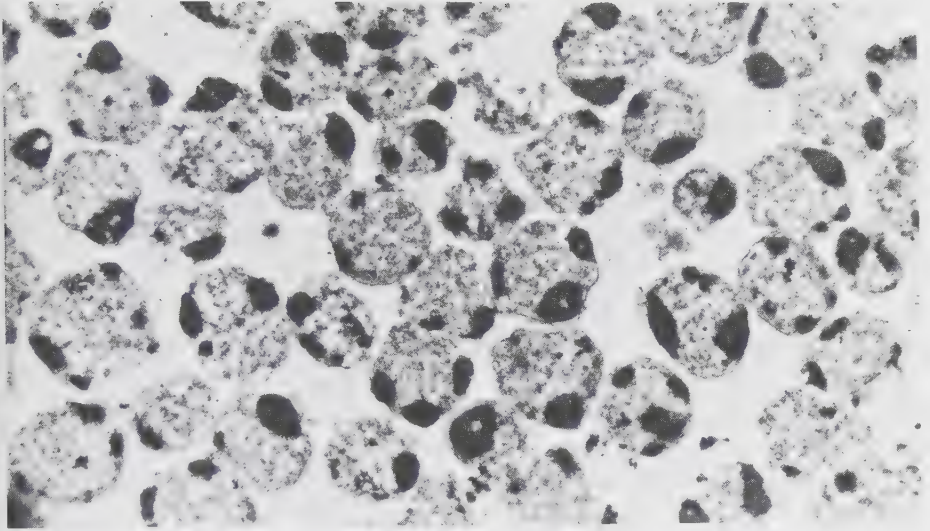


Fig. 2 Nuclei from strain Ax-3 isolated with Triton X-100 and purified by centrifugation through 2.2 M sucrose (x 4500).

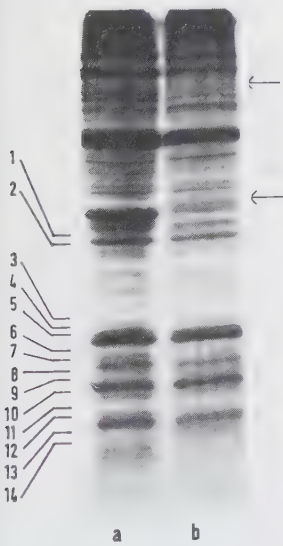


Fig. 3 SDS-PAGE (15%) of nuclei isolated from *D. discoideum*.

a) strain Ax-3
b) strain NC-4

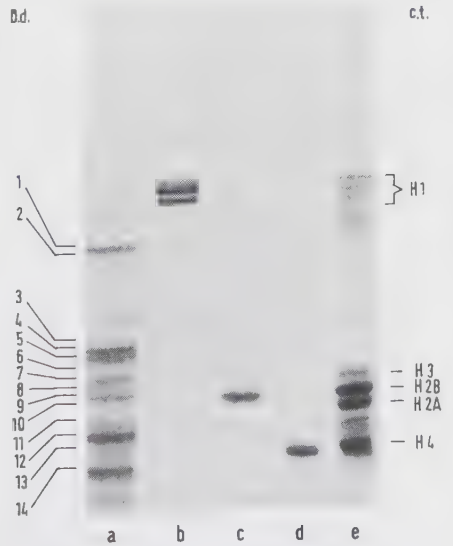


Fig. 4 Comparison of *D. discoideum* (D.d.) BNP (a) and calf thymus (c.t.) histones (b-e) on SDS-PAGE (15%).

2. Isolation of basic nucleoproteins

a) Basic nucleoproteins isolated from vegetative cells

The procedure of Charlesworth and Parish (59) was used effectively to isolate BNP from myxoamoebae of both strains. Some proteolytic activity was present in NC-4 nuclei, as also reported by Chapleo-Charlesworth (93), and the addition of a protease inhibitor was indispensable. Hence, 0.1 mM PMSF was added to the isolation solutions.

The typical electrophoretic pattern obtained from *D. discoideum* BNP is shown in Fig. 4. On SDS gels containing 15% acrylamide, approx. 14-16 bands were visible. All bands, with the exception of band 14, migrated within the same molecular weight range as calf thymus histones (Fig.4). The protein which is thought to correspond to an H1-type histone separated into two bands (numbers 1 and 2) on our gel system. Band 2 showed some variability and was not always present. The other BNP were consistently found.

BNP were also characterised on acid-urea polyacrylamide gels. The method of Panjim and Chalkley (77) was first tested and gave good resolution of the bands (Fig. 5). However, the tubular gels were inconvenient for comparing different samples and for autoradiography. Slab gels made this method polymerised very poorly and so we adopted Hurly's system (79) (Fig. 6). Resolution and sharpness of the bands, especially in the region of bands 2a', 2b' and 3a', 3b', were slightly decreased. On the other hand the pattern was reproducible and a better comparison of the samples was possible. A group of only 5-7 prominent bands could be seen on acid urea gels.

The numbering of BNP on acid urea gels proposed by Chapleo-Charlesworth (93) was retained. This numbering does not correspond to the numbering used on SDS gels.

The extraction procedure of Wang et al. (76) using 5M urea and

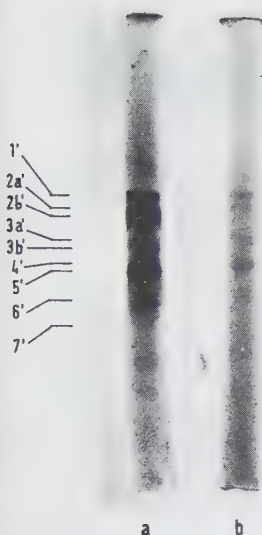


Fig. 5 Comparison of BNP on acid-urea tube gels and isolated according to a) Charlesworth and Parish, b) Wang et al.

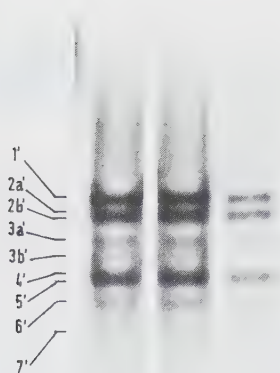


Fig. 6 Acid-urea slab gel of BNP isolated from stationary cells.

increasing salt concentrations at different pH values was also employed. Unfortunately only very small amounts of material were obtained. Electrophoretical patterns of BNP isolated according to Charlesworth and Parish (59) and Wang et al. are compared in Fig. 5. Bands 1', 3', and 5' were still detected and, although of about equal relative intensity, were present in very small amounts. Bands 2a' and 2b' and 4' were lost by this procedure. Only one band, which appears weaker than either 1', 3' or 5', can be seen in this region.

b) Separation of basic nucleoproteins on two-dimensional gels

Basic nucleoproteins were electrophoresed on two-dimensional gels, according to Unger-Ullmann and Modak (81), to determine the relationship between bands of the two gel systems and further resolve the major bands.

Typical 2D gel patterns are shown in Fig. 7. These patterns were reproducible with the exception of bands 9 and 10. Band 9

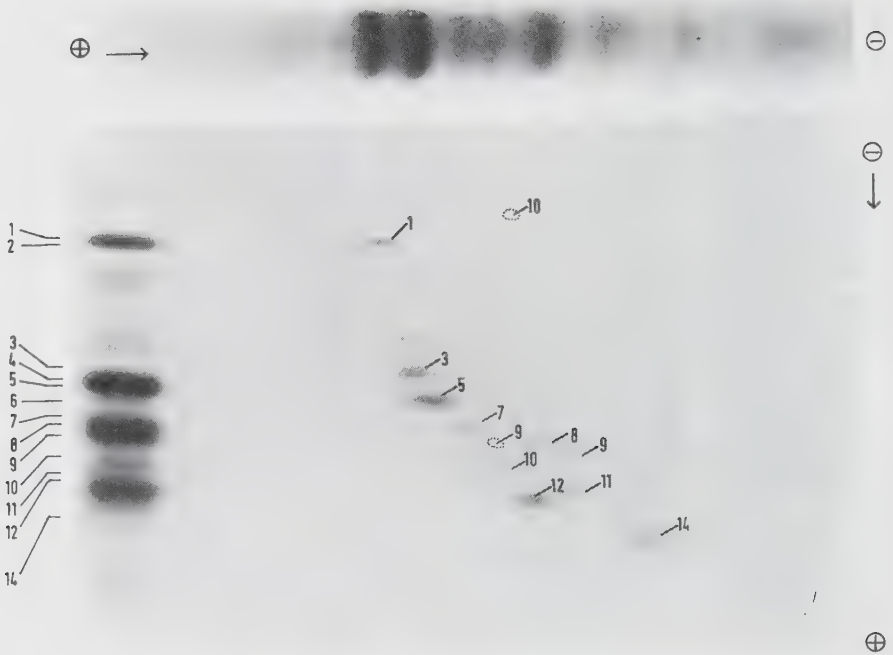


Fig. 7 Two-dimensional gel of BNP. Details in text.

occasionally migrated differently in the first dimension (Fig. 7). Band 10 was once observed to migrate differently in the second dimension, suggesting either dimerisation or aggregation was occurring. The major bands were further resolved into major and minor spots. However, the latter could only be visualized when the first dimension was overloaded with sample (Fig. 31). In such cases the main components of the bands were poorly separated in the second dimension.

The relationship between bands separated on acid-urea gels and SDS-gels is given in Tab. 1. The molecular weight (MW) values were calculated using 15% SDS gels with calf thymus histones as markers (Fig. 4), but must be considered only as reference values. Molecular weights retained for calf thymus histones H1, H2B, H4 were 23,000-21,000, 14,800, 11,200 respectively.

Table 1

Bands on acid-urea	Bands on SDS	Approx. MW on SDS
1'	1, 2	19,000 ; 18,700
2a'	3	15,700
2a' or 2b'	6	14,500
2b'	4, 5	15,500 ; 15,100
3a'	7, 9?	14,400 ; 13,750
3b'	10	13,600
4'	12(a)	12,200
5'	8, 12(b)	13,800 ; 12,200
6'	9, 11	13,750 ; 12,800
7'	14	10,700

Further characterisation of BNP involving amino acid analysis of bands extracted from gels (94, 95, 96) and identification using antisera against calf thymus histones was attempted. However, the results were disappointing. Extraction of BNP from gels for amino acid analysis proved to be technically difficult. Antibodies against calf thymus histone H1 or H2B or H4 reacted unspecifically with all histones of calf thymus and also with the main basic nucleoproteins of *D. discoideum* on SDS gels.

3. Chromatin

Initially, attempts were made to isolate chromatin from nuclei using mechanical methods such as sonication (98, 99, 100) or vigorous agitation in a Vortex mixer (101). No gelatinous material could be obtained. Moreover, the chromatin was certainly sheared by these methods. Consequently, a non-shearing method was sought. Extraction by low (0.1M) (102) or high (0.3M) (100-102) sodium chloride concentrations proved ineffective. Two procedures were found to be satisfactory.

a) Isolation of chromatin according to Comings and Harris (74)

This mild procedure uses 150mM NaCl in 10mM Tris-HCl, pH 8.0. Partial lysis of nuclei was in fact observed and a gelatinous

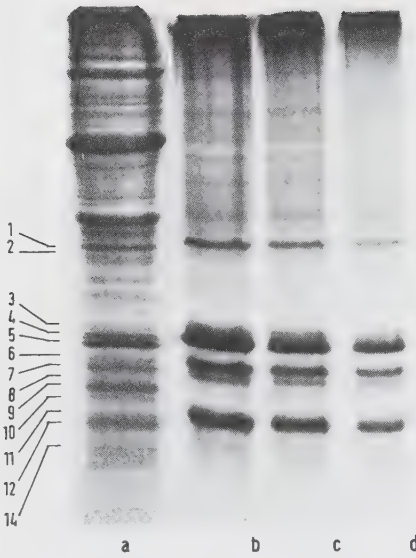


Fig. 8 Comparison of proteins from whole nuclei (a) and "chromatin" (b-d), isolated according to Comings and Harris, on SDS-PAGE.

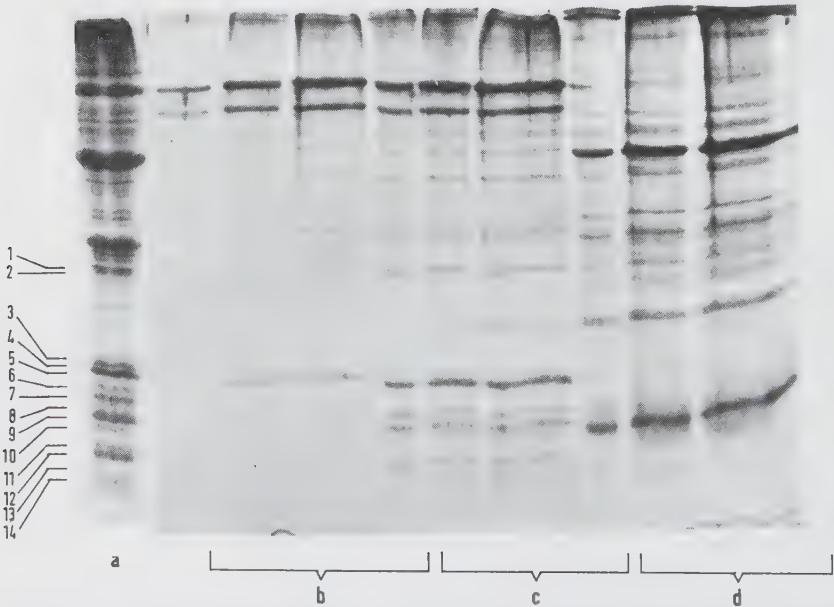


Fig. 9 SDS-polyacrylamide gel of proteins of NaCl/water chromatin isolation. Whole nuclei (a), chromatin (b), supernatants (c) and pellet (d).

material ("chromatin") obtained. It appeared as a light brown colored gel and nucleoli were still present. There was also contamination with whole nuclei.

The protein content of this "chromatin" was examined using SDS gels (Fig. 8). Bands 5, 7 and 12 showed the highest intensity, followed by bands 1, 3, 4, 8, bands 10 and 14 being the weakest. The major basic nucleoproteins were therefore present.

b) Isolation of chromatin by NaCl /water method

Nuclei were washed in 0.15M NaCl, 10mM Tris-HCl, pH 8.0 for very short periods (max. 1 min). Nuclei were lysed by resuspending several times with a pasteur pipette in distilled water ("Material and Methods"). A colorless gel (chromatin) was obtained and contained no nucleoli. These were present in a pellet from which the chromatin could readily be removed using a pipette.

Figure 9 shows the proteins of the three fractions obtained, separated on SDS-13.5% acrylamide gels. Approx. 50 proteins were present in both the supernatant fractions obtained following the 10mM Tris-150 mM NaCl and water extractions. These fractions consisted of nuclear membranes, nucleoplasm, and soluble proteins. Basic nucleoproteins were present: band 10 was extremely strong, followed in order of strength by bands 1, 3, 4-5, 6, 8-9. The chromatin fraction consisted of about 35-40 proteins. Basic nucleoproteins constituted the major part of this fraction, bands 1, 3, 4-5, 7, 8-9, 10, 12, 14 being present. The two strong bands near the top of the gel were probably aggregates, since they were not present in whole nuclei. The insoluble pellet consisted mainly of nucleoli which, however, were poorly if at all solubilized by SDS. Hence, the protein patterns may represent chromatin contamination.

Unfortunately, isolation of chromatin by the NaCl/water method worked effectively only for about 1 year. Subsequently, gelatinous material could be still obtained, but was contaminated with nucleoli and membrane fragments. Changing the sodium

chloride concentration, time of extraction and pH did not overcome these difficulties.

c) Evidence for nucleosomal structure of chromatin

Chromatin is characterised by its regularly repeating structure, the nucleosome, consisting of DNA and histones. Digestion of whole nuclei of *D. discoideum* with micrococcal nuclease produced a series of DNA fragments (61,62) indicating the presence of a nucleosome structure.

Figure 10 shows a gel of DNA fragments obtained following micrococcal nuclease digestion of isolated chromatin, compared with fragments obtained from whole nuclei. Note that chromatin was digested with 75 units of enzyme per ml, instead of 150 units, used for whole nuclei. This was necessary since isolated chromatin was more sensitive to the nuclease. The two monomer subunits (168-185 and 146-152 bp), as well as the multimers are readily detectable in chromatin, indicating the structure has been preserved during isolation. In the absence of nuclease no DNA digestion or shearing to smaller fragments was observed (Fig. 10b).

Examination of chromatin (fixed and stained as described in "Material and Methods") with electron microscopy, revealed a complete absence of contamination by membranes or other nuclear particles. Sections examined under high magnification (Fig. 11) showed thin fibers and globular structures.

4. Nucleoli

We developed a method to isolate nucleoli, the only published method (103), which used sonication, proving unsatisfactory. We initially lysed purified nuclei by homogenization (Sorvall Omnimixer) in Triton X-100, subsequently adding calcium chloride to stabilize the nucleoli. Although nucleoli appeared clean in the phase contrast microscope, electron microscopy showed fibers apparently binding nucleoli together. We modified the method, homogenizing the crude nuclear fraction obtained

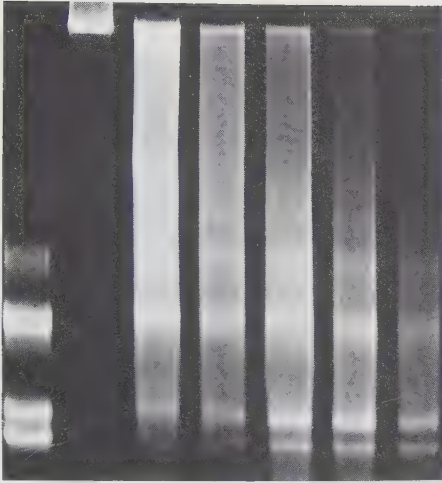


Fig. 10 Polyacrylamide-agarose gel of micrococcal nuclease digests. Nuclei digested 5 min (a), undigested chromatin (b), digested chromatin (c-g): 30 sec, 1, 2, 3, 5 min.

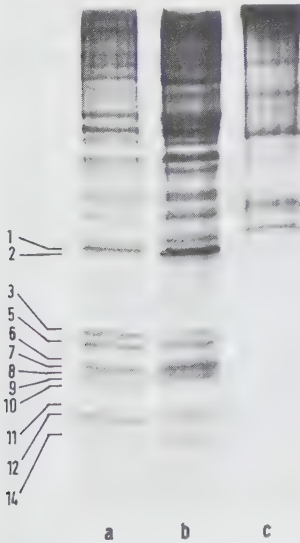


Fig. 12 SDS-PAGE (13.5%) comparing proteins of whole nuclei (a), nucleoli (b) and nuclear membrane (c).

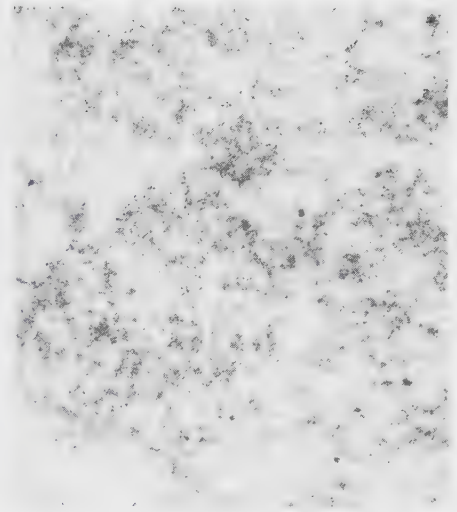


Fig. 11 Thin section of isolated chromatin (fixed in glutaraldehyde, postfixed in 2% OsO_4 , stained in 2% uranyl acetate) (x 12,000).

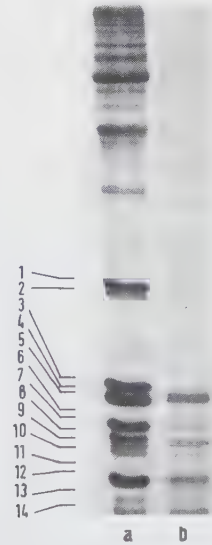


Fig. 13 Proteins of crude nucleosomes on SDS-PAGE (15%).
a) strain Ax-3
b) strain NC-4

after cell lysis with Triton X-100 (see Methods), and obtained pure nucleoli in better yields (104).

Nucleoli electrophoresed on SDS gels showed essentially the same protein pattern as whole nuclei (Fig. 12). Staining intensities indicated that basic nucleoproteins were present at the same concentrations in both samples. Bands 6 and 8 were somewhat stronger in nucleoli.

5. Nuclear Membranes

Pure nuclear membranes (kindly provided by Miss R. Widmer) lacked the basic nuclear proteins (Fig. 12).

6. Nucleosomes

If the basic nucleoproteins we have isolated really have the function of histones we would expect to find them associated with nucleosomes.

a) Crude Nucleosomes

We initially isolated a "nucleohistone" fraction, so-called crude nucleosomes, according to Nelson et al. (75) by digesting nuclei with nuclease.

The crude nucleosome fraction contained predominantly the basic nucleoproteins (Fig. 13). The main bands of crude nucleosomes isolated from strain Ax-3 were 1, 3, 4, 5, 7, 8 and 12. Bands 2, 9, 10, 13 and 14 were present at lower but significant concentrations. Only two bands, 6 and 11, were insignificant in this fraction. Crude nucleosomes, isolated from strain NC-4, contained bands with somewhat different relative intensities: bands 4, 5, 8, 9, 12 and 14 were strong, bands 6, 7, 10 and 13 weaker, bands 1, 2 and 3 present at very low concentrations. Only band 11 was lacking in this fraction.

b) Nucleosome monomers and multimers

Nuclei were digested with micrococcal nuclease and dialysed against Tris-HCl/EDTA buffer. Nuclear debris was removed and

two methods used to fractionate the nucleosomal monomers and multimers of the DNP-particles in the supernatant.

Fractionation on sucrose gradients

We initially attempted to isolate nucleosomes on isokinetic sucrose gradients. The DNP-particles were centrifuged on 5-30% (105), 5-25% (106) or 5-20% (75, 107) w/w linear sucrose gradients, containing 0.1-0.2 mM EDTA. The results were unsatisfactory as only a single unresolved peak (254 nm) at the top of the gradient was obtained. A second large peak could be detected in the middle of the gradients when, according to Woodcock et al. (84), 0.5-1.0 M NaCl was added to the sucrose solutions. Nevertheless, examination of DNA fragments present in the second peak showed that nucleosomal fractionation was still incomplete.

Partial fractionation of nucleosomes was achieved when, according to Staron et al. (83), 0-15% (w/w) linear sucrose gradients were used. Such gradients, containing 10 mM EDTA (pH 7.2) and 0.5 M NaCl, were overlaid with DNP-particles and centrifuged at 95,000 x g for 24 h. Figure 14 A shows the profile of a gradient obtained after centrifuging nucleosomes isolated from 5 min digested nuclei. Fractions I-IV from the gradient were examined for protein and DNA content. Basic and non-basic nucleoproteins were found in all fractions at about the same concentrations (data not shown). Distribution of the DNA isolated from fractions I-IV is shown in Fig. 14 B. A crude but unsatisfactory fractionation of the nucleosomes had occurred. Monomers, for example, were enriched in fraction II, but weaker bands of dimers, trimers, tetramers, and other oligomers were always present.

The resolution of nucleosomes was not improved by increasing digestion times, using higher salt concentrations (1.0 and 2.0 M NaCl), including stronger salts (LiCl), or varying sucrose concentrations.

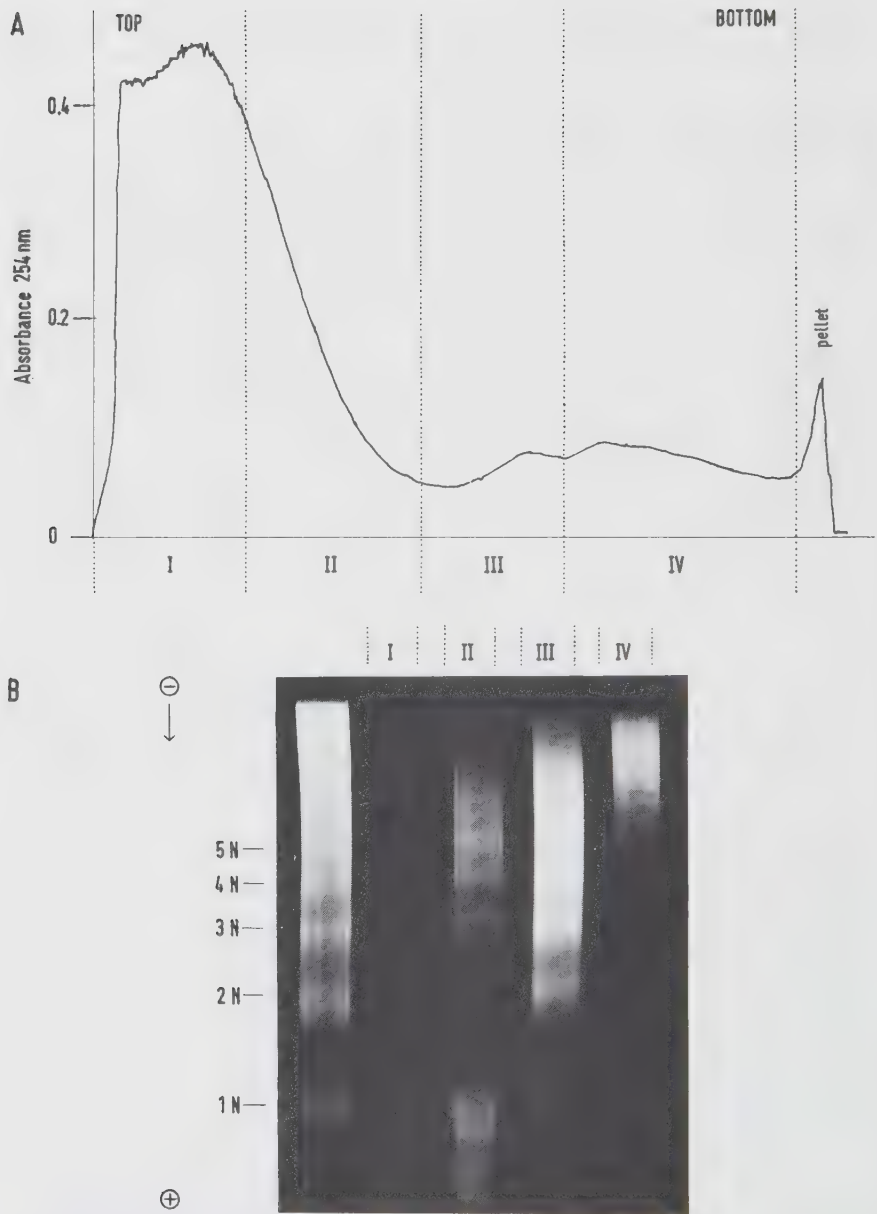


Fig. 14 A) Sucrose density gradient (0-15%) profile of nucleosomes, isolated from 5 min digested nuclei. B) Electrophoretic analysis of DNA from gradient. At the far left an unfractionated sample.

During the course of this work Bakke et al. (62) published a method to isolate polynucleosomes from *D. discoideum* and to fractionate them on 5-30% sucrose gradients, containing 2 mM EDTA, 0.1 mM PMSF. Their results, however, could not be repeated in our laboratory, only two peaks being obtained by this procedure.

Electrophoretic separation of nucleosomes

Nucleosome monomers and multimers were finally fractionated using a gel-electrophoretic procedure based on the methods of Varshavsky et al. (86) and Todd and Garrard (85). Nucleosomes, isolated as described in Material and Methods, were run in a first dimension on a 2% acrylamide/0.5% agarose gel, using Tris-HCl/sodium acetate/EDTA, pH 8.0, as running buffer (Fig. 15/Ia). Electrophoresis in the second dimension was carried out using either 4 or 8% acrylamide, for DNA and protein respectively, with 0.5% agarose and 0.1% SDS. The 0.1% SDS separated DNA from proteins.

Figure 15/Ib shows a second dimension gel stained for DNA. Identification of the DNA fragments (spots) was achieved by using a marker sample (MS) consisting of DNA extracted from freshly isolated polynucleosomes. The monomers, dimers and trimers were separated during the first dimension and were clearly detectable in the second dimension gel. The monomer consists of three major bands, as previously described (61).

Figure 15/Ic shows a second dimension map of the protein components of the fractionated nucleosomes. Three strong bands were detectable in the histone region of the gel. These bands were present in all nucleosome sizes. A large number of higher molecular weight proteins were also detected, mainly associated with the multimers. Clear separation and identification of lower molecular weight proteins was not possible on the second dimension gel. Consequently, proteins and DNA were extracted from the monomers and dimers. The DNP-particles



II

1N

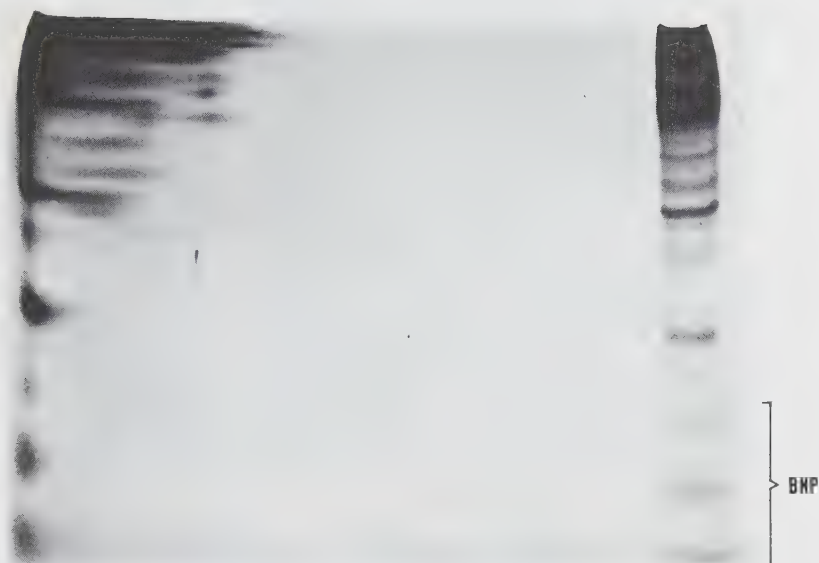


Fig. 15 I: Pattern of DNP-particles isolated from nuclei, following 5 min digestion with micrococcal nuclease, electrophoresed on 2.5% acrylamide, 0.5% agarose and stained for DNA (a). Second dimension mapping of the DNA composition on 4% acrylamide, 0.5% agarose, 0.1% SDS (b). Second dimension mapping of the protein composition on 8% acrylamide, 0.5% agarose, 0.1% SDS (c).

II: Protein pattern of DNP-particles isolated from nucleoli, following 5 min digestion with micrococcal nuclease, electrophoresed on two dimension gels.

MS = marker sample

FMB = fast migrating band

1 N = monomer

2 N = dimer

from nucleoli were also examined on two dimensional gels (Fig. 15/II). Although the BNP were present in the DNP fraction, they did not migrate with DNA in the first dimension, remaining at the top of gel. This suggests their association with DNA differs in nucleoli (see Discussion).

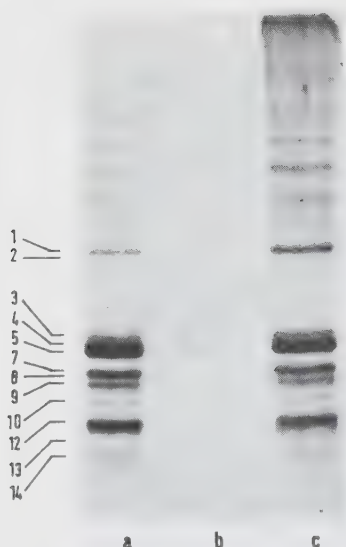


Fig. 16 Proteins extracted from monomers (a), fast migrating DNP-band (b), dimer (c) on PAGE (15%).

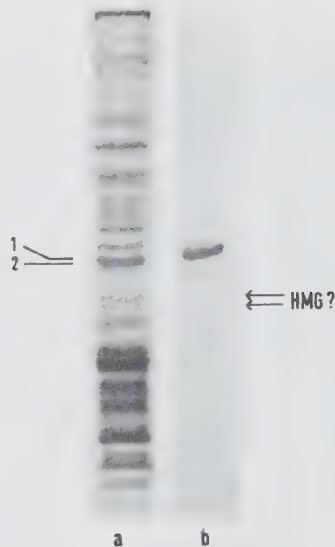


Fig. 17 Comparison of the protein pattern of whole nuclei (a) and the PCA soluble fraction (b) on PAGE (15%).

Electrophoretic patterns of proteins extracted from monomers and dimers

Monomers (1N) and dimers (2N) were identified on first dimension gels by staining with ethidium bromide and cut from the gel. The gel pieces were extracted with SDS and pelleted with acetone (see Methods).

Proteins were analysed on SDS-15% acrylamide gels (Fig. 16). Basic nucleoproteins were the dominant proteins in the monomer fraction. The main bands were 4, 5, 7 and 12, followed by 1, 3, 8-9, 10 and 14. The bands 2, 6, 11 and 13 were not detectable in the monomer fraction. Nine to ten higher molecular weight proteins were also detected. The dimers contained a similar pattern of basic nucleoproteins (Fig. 16). However, band 1 was considerably stronger than in monomers. Bands 2 and 13 were also weakly present. Bands 6 and 11 were still missing.



Fig. 18 SDS-polyacrylamide gel of nuclei isolated at the following stages of the life cycle: growing cells (a), stationary cells (b), very first aggregate (c), rippling (d), streaming (e), early aggregate (f), aggregate (g), tip (h), finger (i), slug (j), mexican hat (k) and culmination (l).

Considerably more higher molecular weight proteins (approx. 20 bands) were present in dimers.

7. The high-mobility-group (HMG) proteins

The low molecular weight bands in the nucleosomes were all apparently basic proteins. It is therefore probable that they have the function of histones in chromatin structure. The HMG proteins also appear in the histone fraction (108-111) and are associated with nucleosomes in higher eukaryotes (112-114). Their function is still uncertain (see Discussion). They are characterised by their solubility in perchloric acid and hence were identified as an impurity of the H1 histone of eukaryotes (115). An attempt to identify HMG proteins from *D. discoideum* was therefore made, studying the electrophoretic pattern of a PCA-extracted band 1 (Fig. 17). Two protein bands (probably double bands) that are only weakly present in nucleosomes and BNP fractions were detected. They are possible candidates for HMG proteins.

Perchloric acid or sodium chloride extraction, according to Goodwin et al (112, 109), was used in an attempt to extract HMG proteins from *D. discoideum* nuclei. No bands could be detected in the expected HMG fraction by these methods.

B. CHANGES IN BASIC NUCLEAR PROTEINS DURING DIFFERENTIATION

The aim of these experiments was to discover whether and at what stages BNP are synthesized during differentiation. This was done by labelling cells with basic amino acids and acetate. Initially we looked for changes in BNP patterns on gels stained for proteins. Nuclei from various differentiation stages were used because the BNP were readily detectable and not contaminated by other proteins on the SDS gels. BNP were extracted from one stage and compared with those of non-differentiating cells.

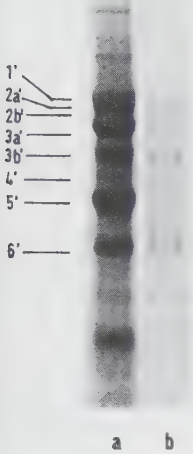


Fig. 19 Acid-urea slab gel of BNP isolated from stationary cells (a) and fingers (b).

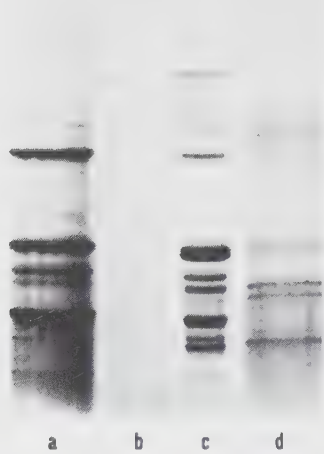


Fig. 20 SDS-electrophoretic pattern of BNP from stationary cells (a), fingers (b), acid insoluble proteins from stationary cells (c) and fingers (d).

1. Nuclear proteins and BNP patterns during differentiation

a) Nuclei

The proteins of nuclei, isolated from different stages of the cell cycle, are shown in Fig. 19. Little variability in the region of the basic nucleoproteins was detectable on SDS gels. All 14 bands were present during entire cell cycle, with the exception of band 1, which disappeared at the slug stage. However, the intensity of the bands varied during differentiation, suggesting changes in the relative concentrations of the proteins occurred. Generally, the lower molecular weight bands 9-14 became stronger as differentiation proceeded. The slug stage was particularly interesting because of its distinct and reproducible pattern. In addition to the disappearance of band 1, bands 9 and 10 increased dramatically in intensity. The possibility that this increase reflected degradation or modification of other proteins, which then migrate identically with bands 9 and 10, could not be excluded.

b) Basic nucleoproteins

The extraction of BNP from differentiating NC-4 cells (aggregates to culmination) proved difficult. Only a minimal amount of proteins could be extracted from large amounts of material (30g cells) by the method of Charlesworth and Parish (59). We extracted BNP from fingers and compared them with BNP from stationary cells. On acid-urea gels (Fig. 19) bands 1', 4' and 5' were present in both stages and seem to have an equivalent charge, as they migrated to the same position on gels. A comparison of the regions of bands 2a', 2b', and 3a', 3b' was difficult. The vegetative cells showed 2 bands which ran very close together in the region 2a', 2b'. The corresponding bands of the finger stage, on the contrary, appeared as clearly separated bands. Bands 3a', 3b' from differentiating cells seemed also to possess different charges, as they migrated further than the corresponding bands from vegetative cells. These variations in charge may reflect secondary modifications of the proteins.

On SDS gels (Fig. 20) the three strongest bands (numbers 1, 5, 7) found in vegetative cells were only weakly present in fingers. The three bands 4, 8 and 14 were still present, but bands 4 and 14 appeared weaker, while bands 6 and 7 were completely lacking in fingers. Band 11 seemed to be a BNP characteristic for fingers, since it was weaker in vegetative cells.

Chromatin structure probably changes during differentiation. DNA-protein interactions were no longer so sensitive to 1M CaCl_2 in differentiating cells and only poor yields of proteins could be extracted by this treatment. The protein fraction which was extracted with CaCl_2 , but was not subsequently soluble in acid, contained BNP contaminated by other proteins. Here the marked differences between BNP of fingers and stationary cells could also be seen (Fig. 20).

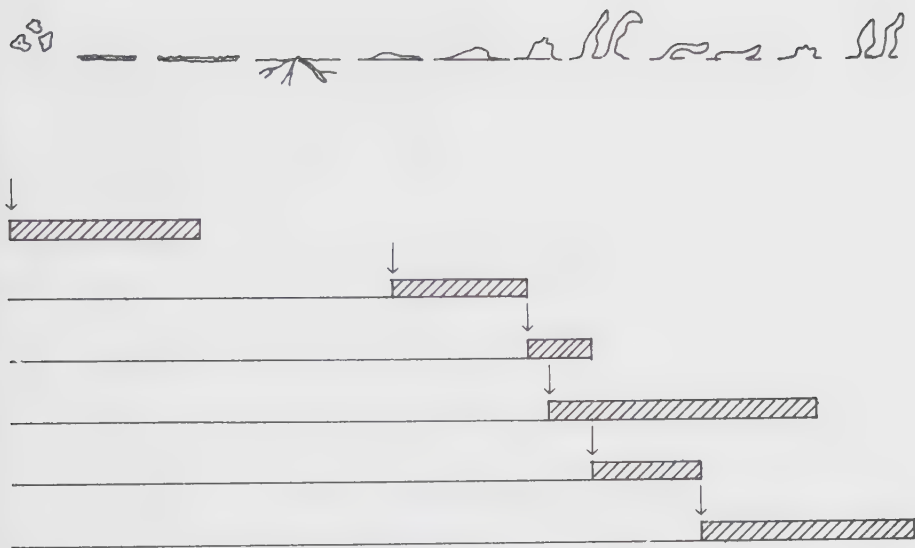


Fig. 21 Schema of short-term in vivo labelling experiments with ^3H -lysine/arginine. Arrow indicates addition of isotope, bars indicate labelling period.

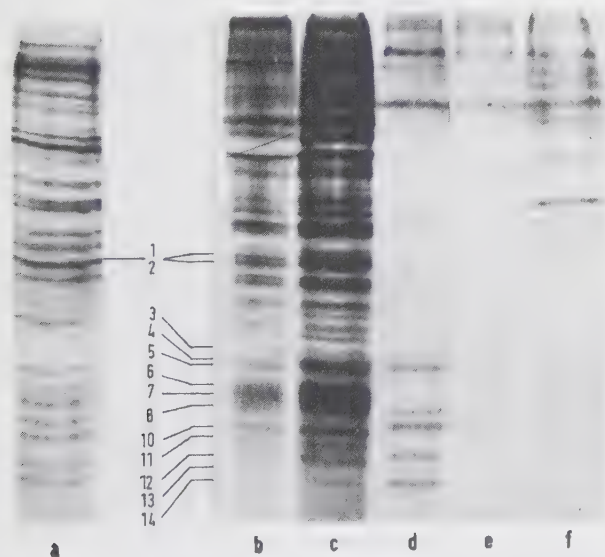
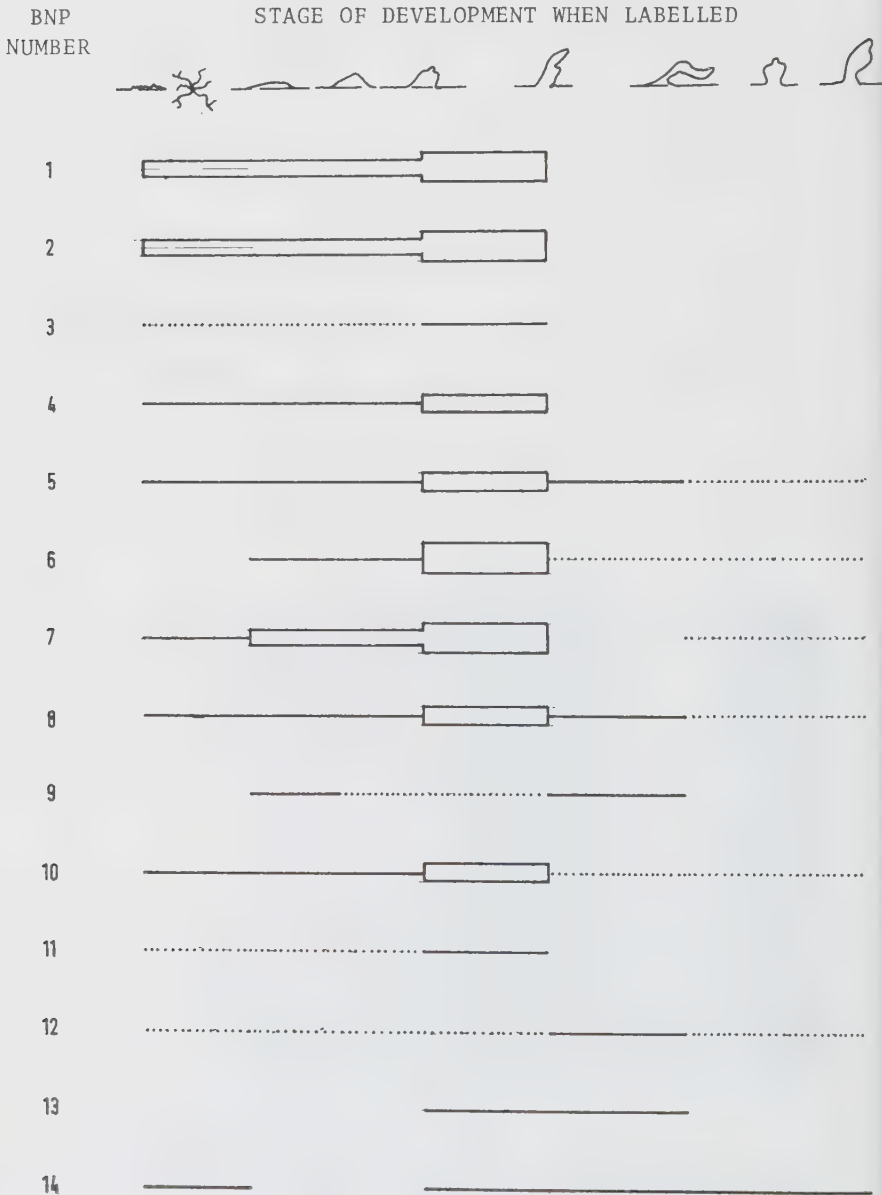


Fig. 22 Fluorograph of proteins labelled with ^3H -arginine/lysine at the following differentiation periods: stationary cells-rippling (a), early aggregate-tip (b), tip-finger (c), finger-slug (d), tip-mexican hat (e), slug-culmination (f).

Table 2 Incorporation of ^3H -lysine/arginine into BNP during differentiation.



2. The synthesis of basic nucleoproteins

a) Labelling with ^3H -arginine/lysine

Cells were labelled with tritiated arginine and lysine to detect synthesis of BNP. Cells (NC-4) grown in liquid culture were plated on membrane filters and labelled at different stages of the differentiation cycle, as schematically shown in Fig. 21. At the end of the labelling period, pure nuclei were then isolated and analysed on PAGE. Incorporated radioactivity was detected by fluorography.

The autoradiographs of the labelled proteins (Fig. 22) showed that protein synthesis occurred during a large part of differentiation. Incorporation of amino acids began at the rippling stage. Dramatic changes occurred at the slug stage and protein synthesis decreased suddenly. Table 2 summarizes the results. Isotope incorporation into BNP reached a maximum between tip and finger stages. The exceptions were bands 9 and 12, which were labelled between finger and slug stages, and band 14, which was labelled between tip and culmination. The four most strongly labelled proteins (bands 1,2,6 and 7) were no longer (or very weakly) synthesized after the finger stage. However, the synthesis of six other BNP continued beyond the finger stage, four of them (numbers 9,12,13 and 14) showing maximum isotope incorporation between the finger and slug stages.

b) Labelling with ^{14}C -acetic acid

Cells were labelled with ^{14}C -acetic acid at various stages of development (Fig. 23). Some of the autoradiographs are shown in Fig. 24. Incorporation of acetate into proteins took place during the entire differentiation cycle. It reached a maximum in the middle stages of differentiation, in particular at the finger stage. A large number of experiments were carried out and these are summarized in Table 3.

Comparison of Tables 2 and 3 shows that the four bands, num-

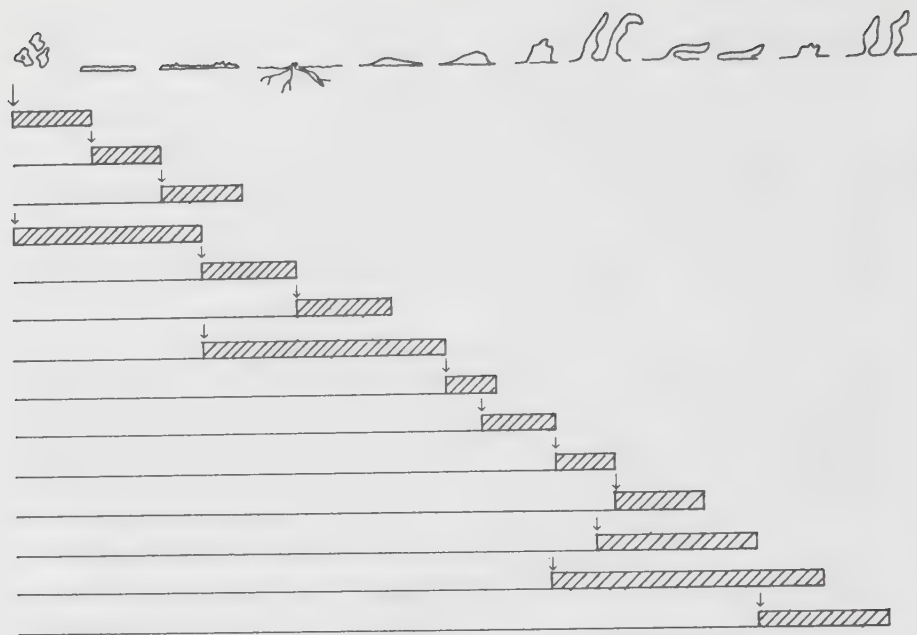


Fig. 23 Schema of short-term in vivo labelling experiments with ^{14}C -acetic acid. Arrow indicates addition of isotope, bars indicate labelling period.

bers 1,2,6 and 7, were strongly labelled both with lysine and arginine and with acetate. On the other hand, bands 3,5,10 and 12 incorporated acetate more strongly than lysine and arginine.

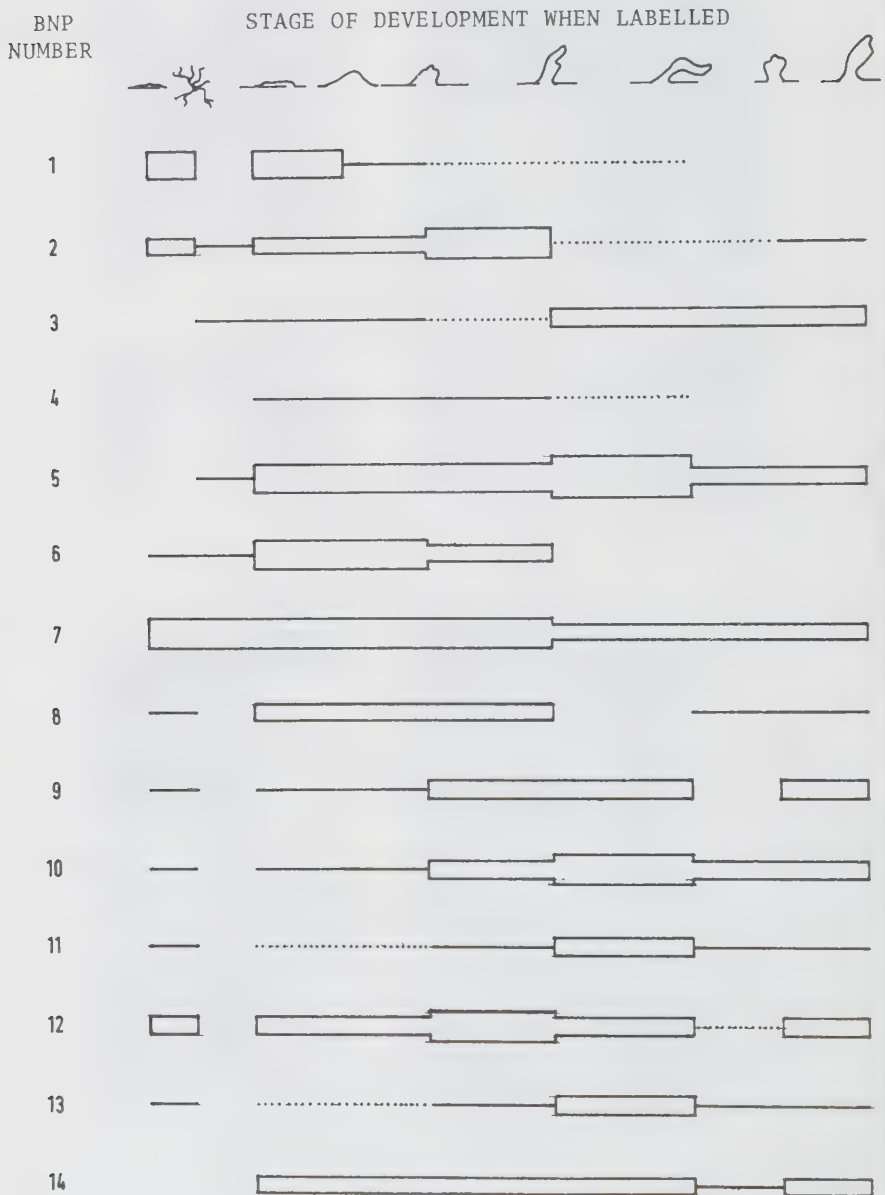
c) Disaggregation experiments

Tips or slugs were disaggregated and allowed to redifferentiate in the presence of isotopes (^3H arginine/lysine, ^{14}C -acetic acid). When cultures had again reached the equivalent differentiation stage, nuclei were isolated and BNP examined for isotope incorporation as before. (In disaggregated slug cells, re-aggregation was until between the finger and pre-culmination stages since the slug stage does not reappear).



Fig. 24 Autoradiographs of nuclear proteins labelled with ^{14}C acetate at various stages of differentiation.

Table 3 Incorporation of ^{14}C -acetic acid into BNP during differentiation.



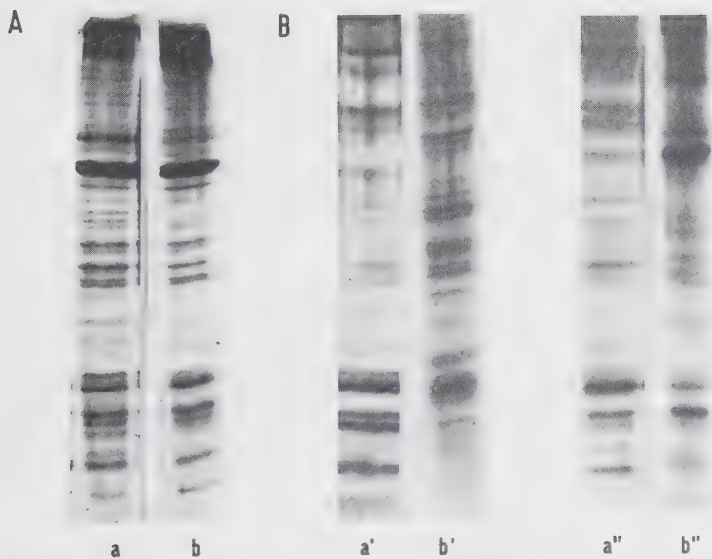


Fig. 25 A) Nuclear proteins pattern from reaggregated tips (a) and non-reaggregated tips (b). B) Autoradiographs of the same samples labelled with ^3H -arginine/lysine (a',b') and ^{14}C acetate (a'',b'').

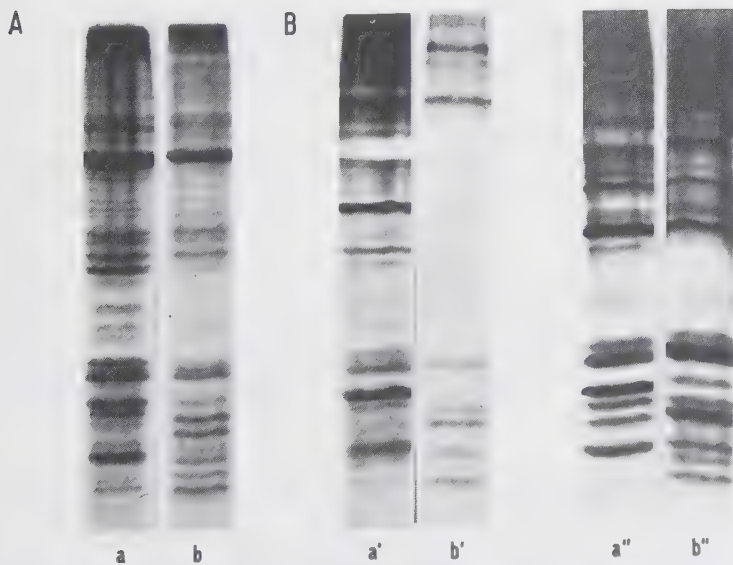


Fig. 26 A) Nuclear proteins pattern from reaggregated slugs (a) and non-reaggregated slugs (b). B) Autoradiographs of the same samples labelled with ^3H -arginine/lysine (a',b') and ^{14}C acetate (a'',b'').

In reaggregated tips five proteins (numbers 2,6,9,10 and 11) had no longer incorporated lysine and arginine (Fig. 25 and Table 4). In other words, their synthesis was inhibited by disaggregation and not reinitiated during reaggregation. A number of BNP (eg. numbers 3 and 5) were BNP that otherwise showed maximum incorporation between the tip and finger stages.

In slugs only 6 BNP were synthesized and only two of these (numbers 5 and 12) were resynthesized during reaggregation (Fig. 26 and Table 4). Bands 2,6 and 11 were also not labelled, suggesting their resynthesis was not induced (or that the newly synthesized molecules are rapidly broken down). The synthesis of BNP 1,3,7 and 8 was reinitiated during reaggregation of disaggregated slug cells.

Acetate labelling confirmed the results obtained with arginine and lysine. Exceptional were bands 10 and 14 which, although not incorporating amino acids in reaggregating slug cells, were

Table 4 Incorporation of ^3H -lysine/arginine in BNP from reaggregated tips and slugs (see text).

TIPS	BNP number:													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	+	+		+	?	+	+	+	?	?	+		+	
Reaggregated	+		+	+	+		+	+				+		+

SLUGS	BNP number:													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control					+		?	?	+	?		+	+	+
Reaggregated			+		+		+	+				+		

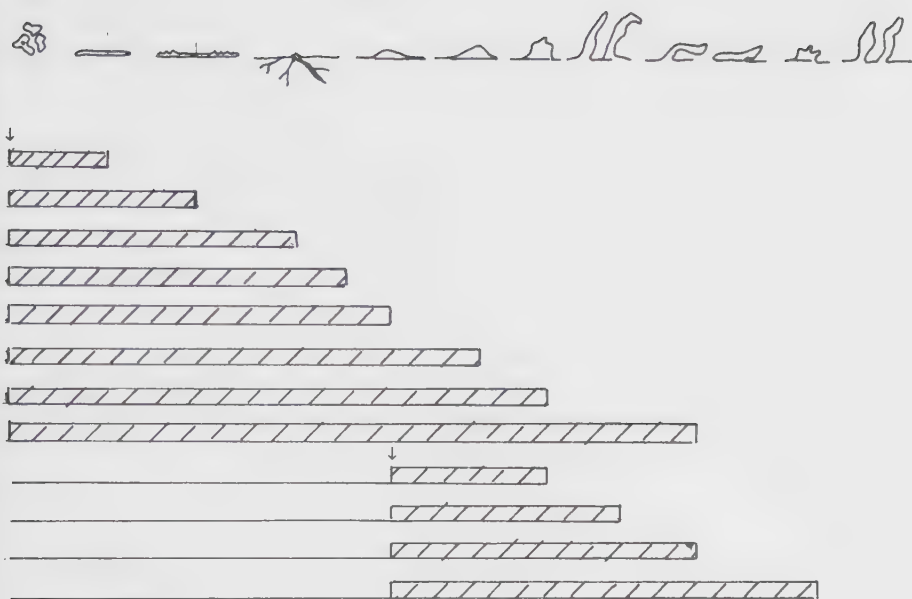


Fig. 27 Schema of long-term in vivo labelling experiments with ^{14}C -acetic acid. Arrow indicates addition of isotope, bar indicate labelling period.

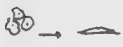
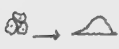
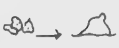
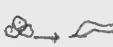
labelled with acetate. This suggested they were being acetylated (see later).

BNP patterns were similar in control and reaggregated tips, except for the relative concentrations of bands 3 and 14. The unique pattern found in slugs was no longer detected following reaggregation to fingers-precumination stage. This may be because the slug stage is circumvented during reaggregation.

d) Long-term labelling of basic nucleoproteins

The possibility that some of the newly synthesized BNP are broken down again when their synthesis ceases was examined using long-term labelling.

Table 5 Incorporation of ^{14}C -acetic acid into BNP during long-term labelling (see Methods).

LABELLING PERIOD :	BNP number:													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
 → 	+	+	+	+			+	+				+		
 → 	+			+	+	+	+	+	+	+		+	+	?
 → 	+			+	+	+	+	+		+		?	+	
 → 	+			+	+	+	+	+		+			?	
 → 	+			+	+	+	+	+	?		?			?
 → 	+				+	+	+	+	+	+	?	+	+	+
 → 	+	?	?	+	+	+	+	+	?	+	?	+	+	+
 → 	+	+	+	+	+	+	+	+		+	+	+	+	+
 → 	+	+	+	+	+	+	+	+		+	+	+	+	+

+ = weak labelling ; ++ = strong labelling ; +++ = very strong labelling.

The design of the experiments is shown in Fig. 27. The results are summarized in Table 5. The presence of ^{14}C -acetate from $t=0$ failed to label some BNP which normally incorporate isotope during short-term labelling. Since these BNP (numbers 3, 11 and 14) were synthesized rather late (Tables 2 and 3), dilution of radioactive pools may be responsible. All BNP incorporating acetate, with the exception of number 9, remained labelled to the final stages of differentiation. Hence, newly synthesized molecules of BNP 9 were apparently turned over rapidly. Since some BNP are acetylated until culmination (Table 6, see later), the acetate labelling provided no information about their turnover. We did not, however, repeat the experiments with arginine/lysine because of the problems of radioactive pool size which became apparent during acetate labelling (see above).

3. Modification of basic nucleoproteins

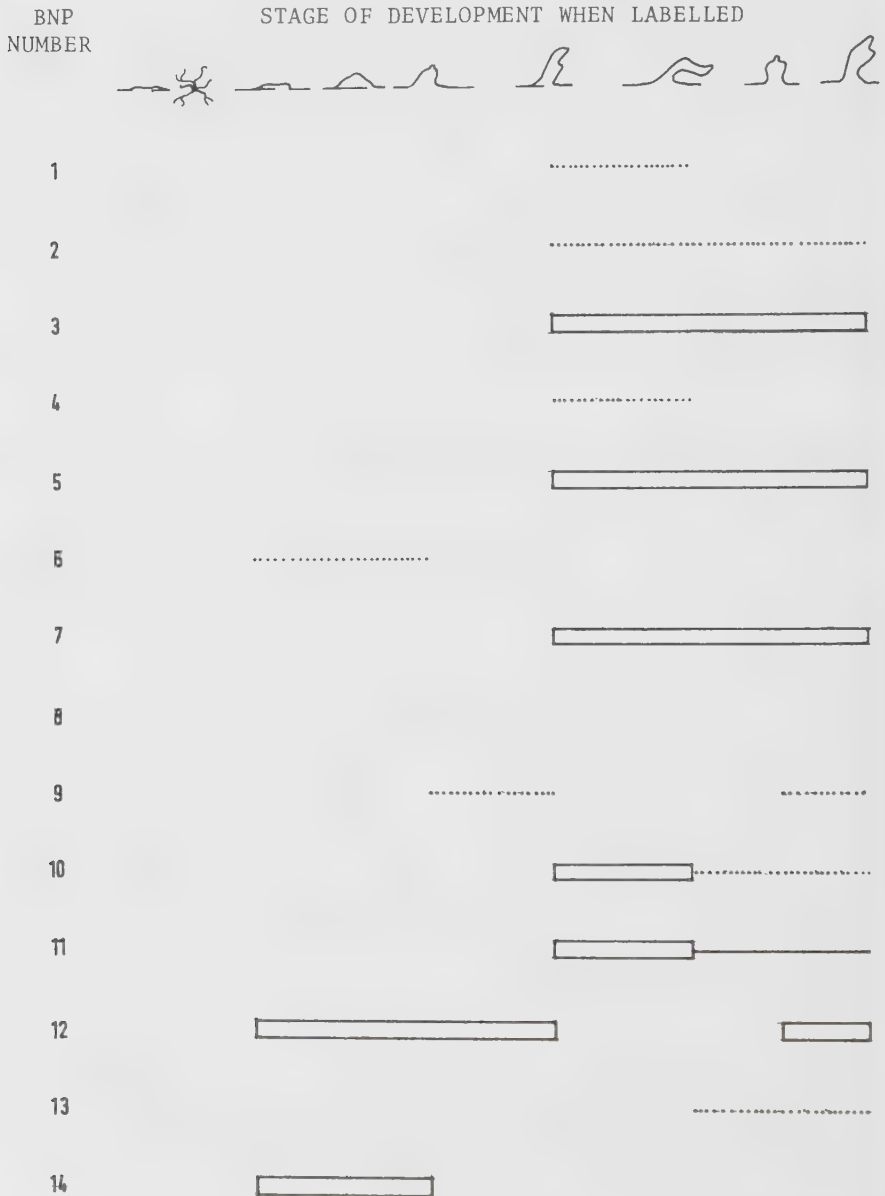
The aim of these experiments was to discover whether and at what stage BNP were modified during differentiation. Only two of the four known secondary modifications of BNP were examined: acetylation and phosphorylation.

a) Acetylation of basic nucleoproteins

Usually, in vivo acetylation of histones was investigated using labelled acetic acid (116-119). However, since acetate is also incorporated into amino acids, we estimated acetylation by comparing labelling patterns with acetate with those obtained with lysine/arginine (Tables 2 and 3). Basic nucleoproteins which, although at certain stages incorporating arginine and lysine, were labelled at other stages exclusively by acetate were considered acetylated.

The results compiled from Tables 2 and 3, are shown in Table 6. Acetylation was positively identified in six BNP. Based on relative strengths of labelling with arginine/lysine and with acetate, acetylation of five of these BNP (numbers 5,7,9,12

Table 6 Acetylation of BNP during differentiation. The results are compiled from Tables 2 and 3 so that acetylation in the presence of synthesis is not indicated (see Text).



and 14) probably also occurred when they were being synthesized. The remaining BNP were at best only weakly acetylated. Acetylation continued until the late culmination stage. (Nuclei could not be isolated from later stages).

Sung et al. (116) recommended the use of cycloheximide to distinguish incorporation of amino acids in *de novo* synthesised molecules from acetylation of the proteins. We labelled differentiating cultures with acetate in the presence of cycloheximide (400 µg/ml). However, incorporation was very low and the results ambiguous.

b) Phosphorylation of basic nucleoproteins

In vitro

Nuclei were incubated with (γ -³²P)ATP. Incorporation into acid insoluble products reached a maximum after 60 sec and then began to fall, levelling off at about 20% of original incorporation after 60 min (data not shown).

Strongly phosphorylated were BNP 1 or 2 and 4 or 5. Since the bands were broad on autoradiograms it was difficult to be sure which member of each protein pair was labelled. The phosphorylation of BNP remained constant during the normal labelling period (10 min) and then began to diminish (Fig. 28). However, although band 4/5 was no longer labelled after 60 min, the activity of band 1/2 had decreased only slightly (Fig. 29).

No changes in labelling patterns were observed in the presence of RNAase, actinomycin D, spermine or spermidine (data not shown). Treatment of gels with hot TCA (90°C) before exposure did not change the pattern. Interestingly, when calf thymus histones were added they were not phosphorylated.

The labelling of nuclei and nucleoli isolated from vegetative cells was compared. In nucleoli labelling corresponding to bands 7 and 10 was stronger and a protein between bands 2 and 3 (denoted "X") was strongly labelled (Fig. 30).

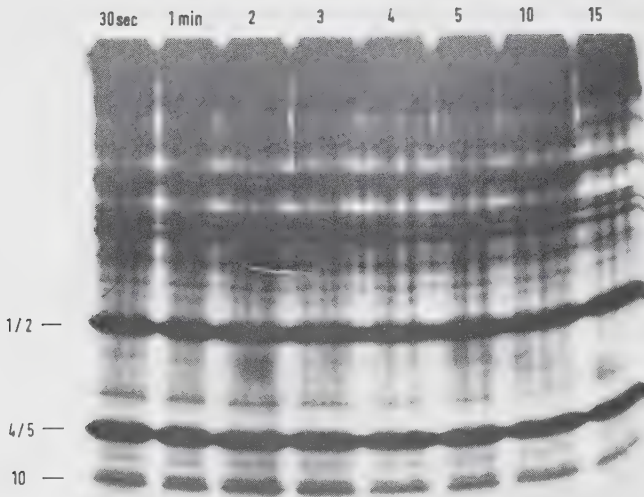


Fig. 28 Autoradiographs of nuclear proteins labelled in vitro with (γ - ^{32}P)ATP for various times.

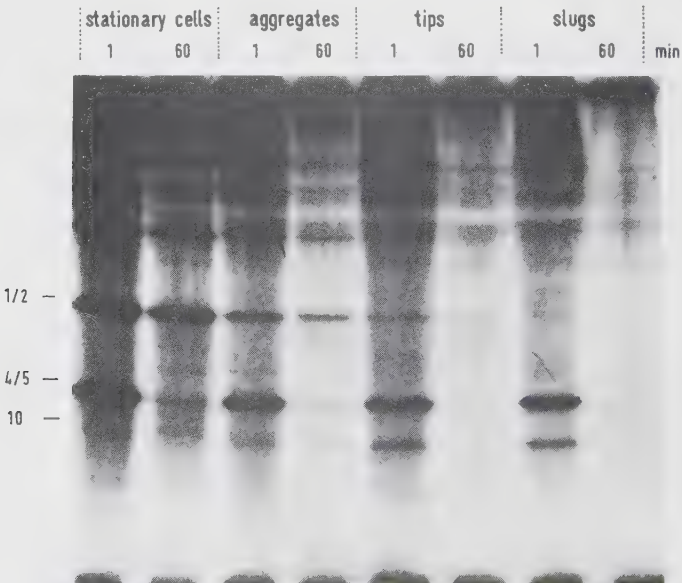


Fig. 29 Autoradiographs of nuclear proteins from four developmental stages labelled in vitro with (γ - ^{32}P)ATP for 1 min and 60 min.

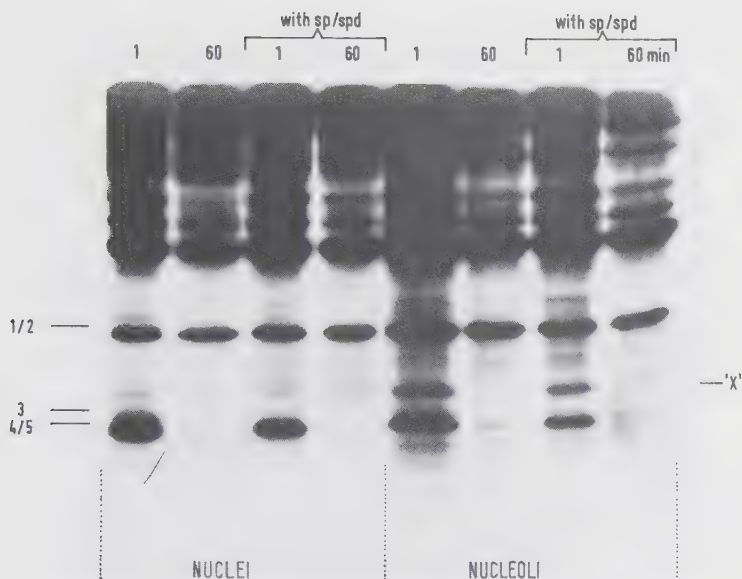
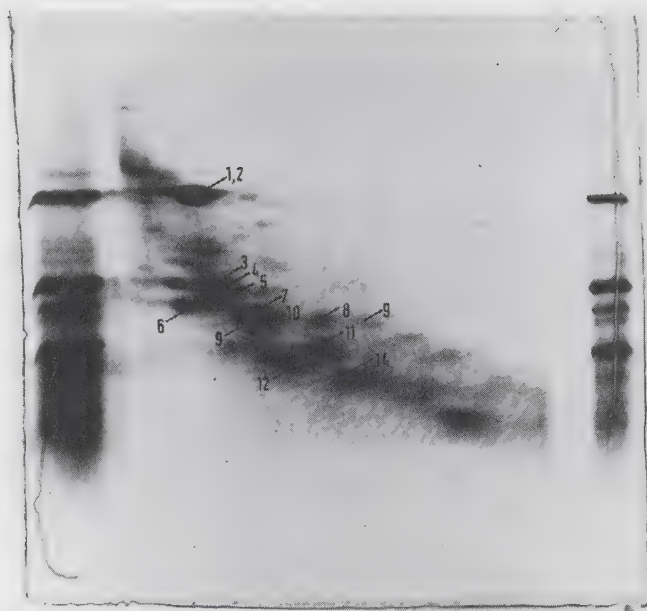


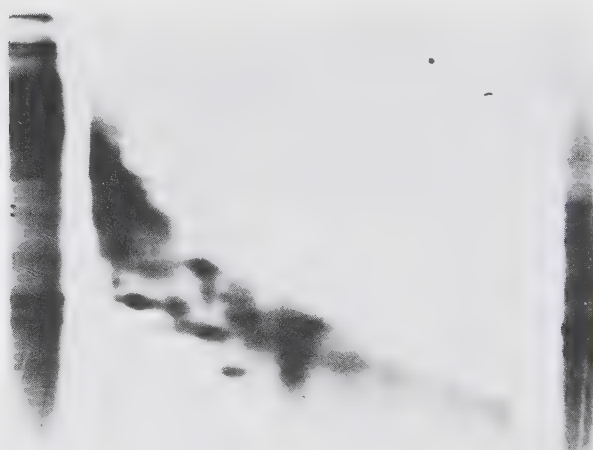
Fig. 30 Autoradiographs of nuclear and nucleolar proteins labelled in vitro with (γ - ^{32}P)ATP for 1 min and 60 min with or without spermine/spermidine (sp/spd).

Nuclei from vegetative cells were phosphorylated, the BNP extracted and separated on two dimensional gels. A large number of labelled spots were detected by autoradiography (Fig. 31). Comparison of radioactive spots with protein staining indicates that the phosphorylated proteins migrated less in the first dimension. A line of four radioactive spots for example, were associated with band 4/5. This presumably reflects the number of phosphate groups per protein molecule. Since phosphorylation was associated mostly with minor or even undetectable spots, only a small proportion of BNP molecules were phosphorylated. Minor spots which were not labelled may represent other modification (eg. acetylation) or have been fully phosphorylated *in vivo*. The possibility that some minor proteins were contaminants of the major BNP is impossible to exclude without amino acid analyses.

A



B



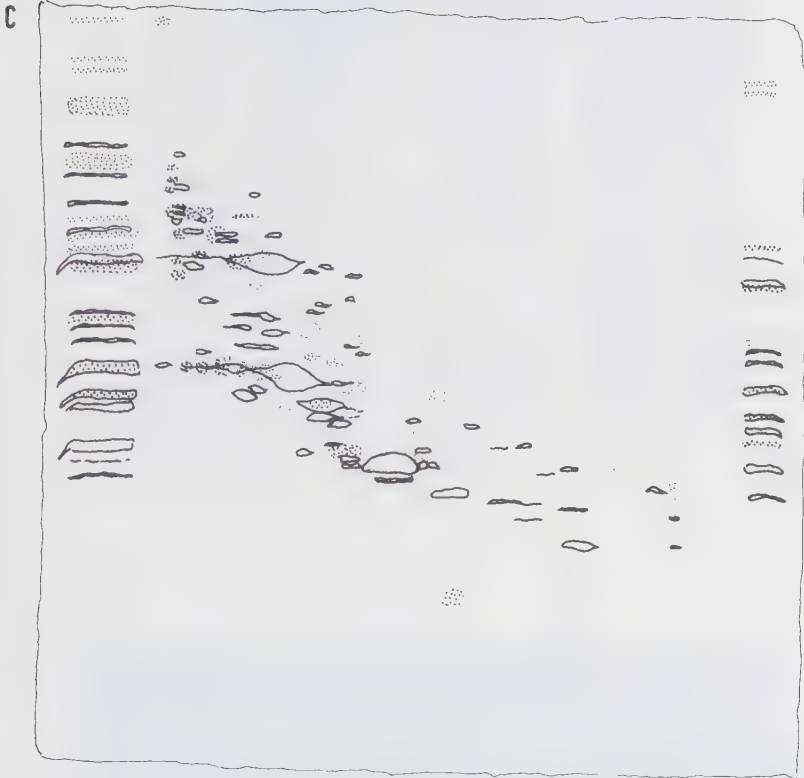


Fig. 31 A) Pattern of BNP isolated from nuclei, following in vitro labelling with (γ - ^{32}P)ATP, on two dimensional gel. B) Autoradiograph of the same gel. C) Graph summarizing A) and B).

— protein position
 radioactivity

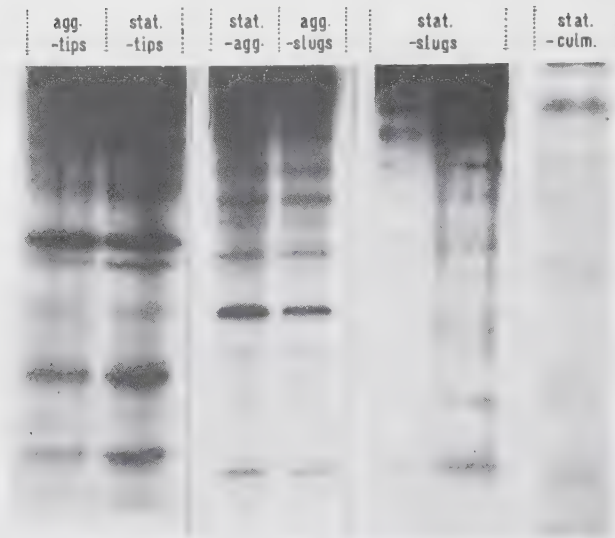


Fig. 32 Autoradiographs of nuclear proteins labelled in vivo with inorganic ^{32}P at various stages of differentiation.

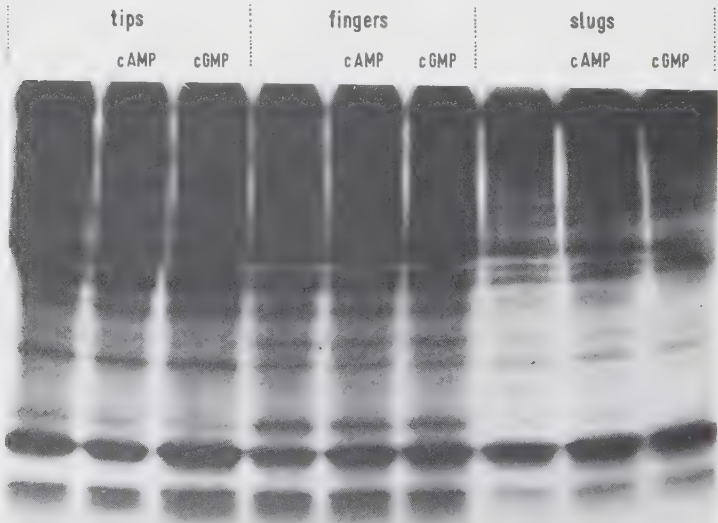


Fig. 33 Autoradiographs of nuclear proteins from 3 developmental stages, labelled in vitro with $(\gamma\text{-}^{32}\text{P})\text{ATP}$, with or without cyclic nucleotides (10^{-6}M).

Nuclei were isolated from various differentiation stages and changes in *in vitro* phosphorylation of BNP examined (Table 7). The most strongly labelled BNP were numbers 1/2 and 4/5, however, band 10 also became labelled during aggregation (Fig. 33, see later). No changes in the dephosphorylation rates of labelled BNP was found during differentiation.

I n v i v o

Differentiating cultures were labelled at various times with inorganic ^{32}P . Gels were routinely treated 10 min with hot TCA (90°C) before exposure to remove RNA and DNA. However, as judged by staining intensity, all proteins were also to some extent lost. No changes were observed in phosphorylation patterns except the bands were weaker. The pattern of BNP labelling was very different from that obtained *in vitro* (Table 8). In particular, the strong labelling of numbers 4/5 and 10 was totally lacking (Fig. 32). Only the phosphorylation of BNP 1/2 was consistent with the *in vitro* results. Phosphorylation of bands 7 and 8 was much more pronounced *in vivo*.

Effects of cyclic nucleotides i n v i t r o

The presence of cGMP or cAMP (usually 10^{-6}M) during *in vitro* labelling had no notable effect on phosphorylation (Fig. 33). When examining the effect of nucleases on phosphorylation, we found that prior incubation of nuclei at pH 5.0 strongly inhibited the subsequent *in vitro* phosphorylation at pH 7.0 (Fig. 34 a). The presence of cGMP and cAMP did not reduce this inhibition. However, when nuclei had been incubated with DNAase I, phosphorylation was largely restored and the cyclic nucleotides again without effect (Fig. 34 e-g). Treatment with DNAase II partly restored *in vitro* phosphorylation and this was further increased when cAMP and, in particular, cGMP were present (Fig. 34 b-d). Phosphorylation in the region band 10 differed somewhat according to which nuclease was used.

Table 7 Incorporation of ^{32}P into BNP following incubation of nuclei from different developmental stages with (γ - ^{32}P)ATP.

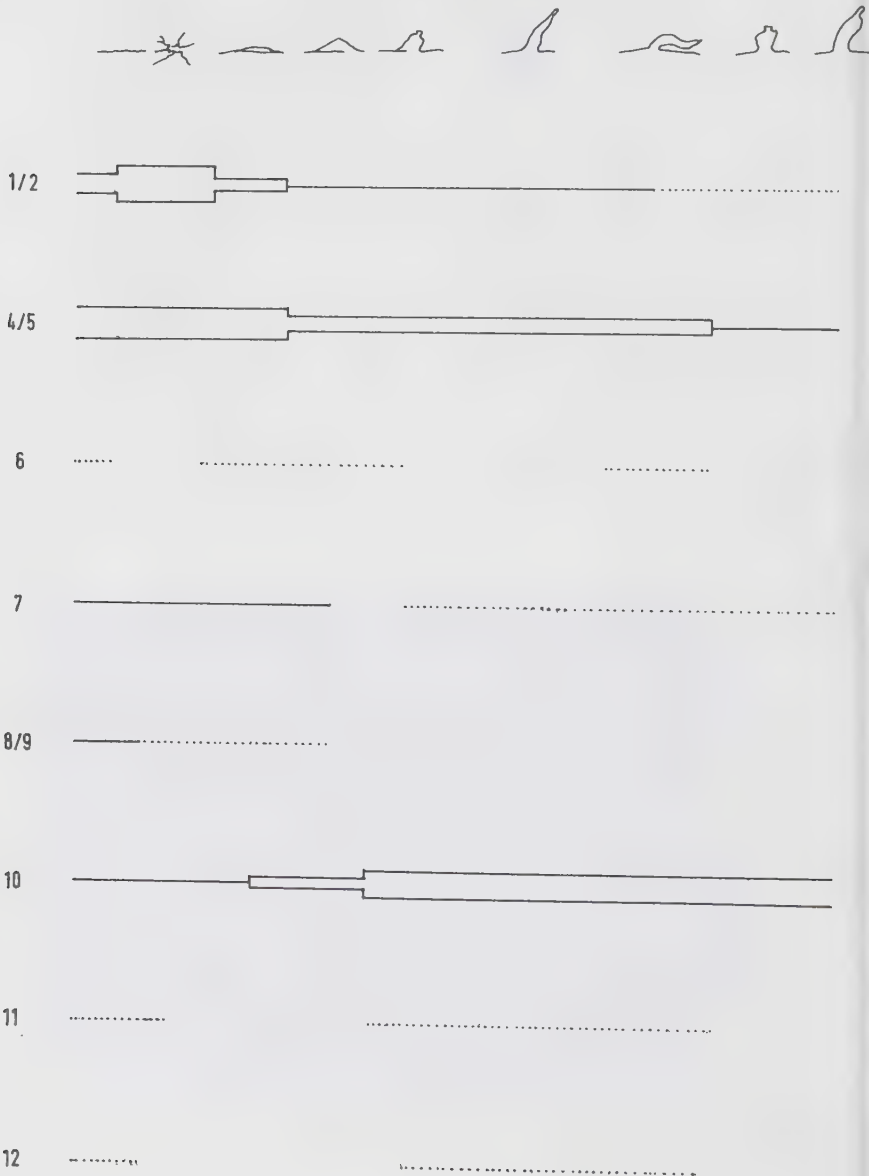
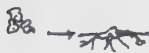
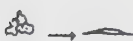
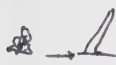


Table 8 I n v i v o incorporation of inorganic ^{32}P into BNP during differentiation.

LABELLING PERIOD :	BNP number:													
	1/2	3	4	5	6	7	8	9	10	11	12	13	14	
	+			+	+	+								
	+				+	+						+	+	
	+				+	+	+							
	+					+	+							
	+				+	+								
	+					+	+							
	+					+	+							
	+					+	+					+	+	

+ = weak labelling ; ++ = strong labelling ; +++ = very strong labelling.

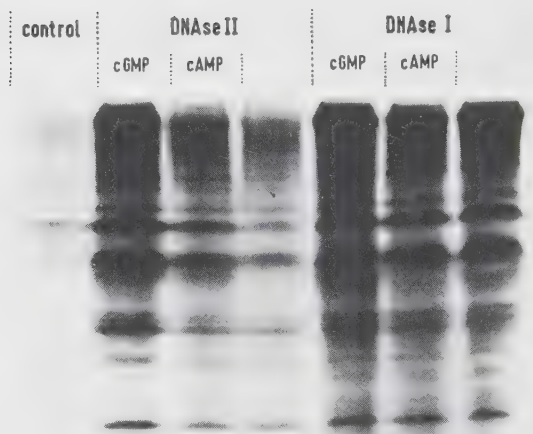


Fig. 34 Effects of nucleases on in vitro phosphorylation in the presence or not of cyclic nucleotides (see text).

IV. DISCUSSION

Evidence that the BNP are histones

We identified 14 BNP using SDS-PAGE and these were probably separated on the basis of amino-acid composition. The additional resolution obtained with 2D electrophoresis presumably reflected secondary modifications of the proteins.

If the BNP have the function of histones we would expect to find them in the nucleosomes. All (except number 11) BNP were present in chromatin and a crude nucleosome fraction.

We initially attempted to purify mononucleosomes on sucrose gradients. The experiments were unsuccessful due to nucleosome aggregation and, although widely used, the resolving power of this technique is in any case rather low (117). Subsequently, we were able to isolate nucleosome monomers and multimers with PAGE. Ten of the BNP were found associated with the mononucleosomes. BNP 2, which like number 1 has the properties of an H1 histone, was present in nucleosome dimers and was probably lost from monomers during digestion. BNP 13 was also only found in dimers and is probably not a core histone. Bands 6 and 11 were absent from nucleosome monomers and dimers and so are probably not histones.

Consequently, we believe twelve of the BNP have histone-like functions although proof of their histone character awaits amino-acid analysis. Unfortunately, we were unable to extract adequate amounts of proteins from gels for analysis, although a variety of techniques were employed. We also induced antibodies against purified calf thymus histones in the hope of identifying similar antigenicity among the D. discoidium BNP. However, for unknown reasons, the antibodies obtained were not specific for individual histones. Hence, although we believe the 12 BNP in nucleosomes are probably histones, we continue to refer to them as "Basic Nuclear Proteins" throughout the Discussion.

Histone Variants

In all organisms so far examined the histone genes are reiterated and clustered. Each of the five different histones is present per repeat unit (118-124). Since they are highly repeated, the histone genes would be expected to be polymorphic. This is evidenced at the protein, in particular for H1, H2A and H2B (125, 126, 127), and DNA level (124). Histone variants may be stage-specific (126) or uniquely associated with highly differentiated cells (128,129).

The 12 BNP from *D. discoideum* associated with nucleosomes may represent histone variants. The relationship between these proteins and the five classical histone groups awaits amino acid analysis. At present we only know that proteins 1 and 2 are equivalent to the H1 histones (60).

Histone variants are often tissue specific (13) although it is not known whether this specificity extends to gene association. It is perhaps relevant that we found the pattern of BNP associated with nucleoli almost identical to that of whole nuclei and to chromatin from which nucleoli had been removed. Nevertheless, the chromatin of *D. discoideum* does have a unique structure (103) and we observed that the BNP are more readily removed from nucleoli than nuclear nucleosomes during electrophoresis. These differences may result from histone modifications or non-histone proteins unique to nucleoli. We were unable to obtain enough material to examine nucleoli BNP with 2D PAGE.

High Mobility Group Proteins

HMG proteins were originally isolated as impurities in the H1 fraction (108, 115) and are present in monomer nucleosomes from rabbit thymus and duck erythrocytes (112, 130, 131). They may be one of the factors responsible for conferring a DNAase I - sensitive structure on active genes (132).

HMG proteins account for only 1-2% by weight of the histone content of chromatin (108) and, hence, the 12 BNP in D. d i s c o i d e u m nucleosomes are not likely candidates. The perchloric acid-soluble BNP ("H1 fraction") did contain some weak bands in the molecular weight range of HMG proteins. When nuclei from rabbit thymus, for example, are treated with 0.35 NaCl, HMG proteins are extracted and can be separated from other proteins by treating with 2% TCA. The HMG proteins remain soluble and are then precipitated with 10% TCA (108). In D. d i s c o i d e u m the major proteins precipitated by TCA are BNP 1 and 2. This is interesting since the amino-acid composition of the perchloric acid soluble ("H1") proteins resembles that of HMG proteins (60).

BNP synthesis

The bulk of histone synthesis is thought to be synchronized with DNA synthesis in the cell cycle (133-138). Nevertheless, some histone synthesis has been reported to occur at other stages of the cell cycle (eg. 135, 139) and during erythrophoiesis (140) and oogenesis (141) it is known to be uncoupled from DNA synthesis. In spite of initial reports to the contrary (142, 143), it appears that both old and new histones cover the newly replicated DNA and the newly made histones are distributed at random in the genome (144, 145). In the absence of DNA synthesis, newly synthesized histones would still presumably become associated with DNA. Whether they form new nucleosomes with stretches of naked DNA or are incorporated into existing nucleosomes is not known. If the nucleosome consisted exclusively of eight histones and 140 base pairs, the histone: DNA ratio would be 1.2:1. However, the ratio in, for example, dry pea embryo is 3.3:1 (146). A spectrum of particles may exist along the DNA with ratios from 0.6:1 up to 3:1 (147), the binding of additional histones to chromatin core particles serving to stabilize local DNA:histone interactions and inhibit DNA replication and transcription. An increase in the number of nucleosomes along the DNA fibre and their histone content may be an important means of repressing

the genome. Genetically dormant systems such as resting seeds (146) and fully differentiated cells (148) certainly do have high histone:DNA ratios.

The BNP synthesis we detected in *D. discoideum* may be related to DNA synthesis and/or genome repression. Synthesis of histone variants, controlled by the developmental programme, might also be involved in gene activation. Zada-Hames and Ashworth (65) found mitotic cell division together with nuclear DNA synthesis at two distinct periods in the life cycle of *D. discoideum*. The first period was during the first 10 hours of development until the earliest visible signs of aggregation occurred (rippling). The number of cells (in NC-4 cultures) increased 10-24% during this time. However, no division occurred during the preaggregation stage in stationary phase cells which are already arrested in G2 (65). The second period of mitotic activity began at tip formation, reached a maximum at the slug stage and decreased during early culmination. There was a 15-20% increase in cell number and labelling showed 12-13% of the cells had passed through the S phase (65). Disaggregation experiments indicated the second mitotic period is a once and for all event in differentiation, and studies with mutants that it is not a random process.

During the first mitotic period radioactive acetate, lysine and arginine were incorporated into many of the BNP. However, labelling was relatively weak. Bands 4 and 5 presented an enigma; although weakly incorporating lysine and arginine, they were not labelled by acetate. This underlines the problems associated with labelling when amino-acid pool sizes are not known. Moreover, radioactivity in a band gives us no clear indication of the relative amount of newly synthesized molecules. Since the various BNP contain variable amounts of lysine and arginine, comparison of synthesis between bands based on strength of labelling is precarious. Nevertheless, if a protein is weakly labelled both by the amino-acids and by acetate then synthesis is probably minimal. Strong labelling with acetate

with weak lysine/arginine incorporation may indicate a weakly basic protein or, more likely, acetylation (see later). Band 12 was such an example.

The strength of labelling during the first mitotic cycle bore no relation to the relative concentrations of the individual proteins.

At mid-aggregation all BNP (with the possible exception of band 12) were incorporating the isotopes. The synthesis of 8 of the BNP ceased or was dramatically reduced at the finger stage. However, the synthesis of 6 BNP continued beyond the finger stage and four of them exhibited their strongest incorporation during this period. DNA synthesis reaches a maximum in slugs (65) and so, if all the new BNP are required because of this DNA synthesis, they are synthesized somewhat earlier than expected. We assume, perhaps incorrectly, that since all BNP are present in vegetative cells they must all be synthesized when cell division occurs during differentiation. Although less cells pass through the S phase in the second than in the first mitotic period, labelling of BNP is much stronger during the later stages of differentiation. This may, however, reflect amino-acid pool sizes rather than a "requirement" for BNP.

The relative strength of labelling with lysine and arginine among the various BNP differed at different developmental stages. Except for bands 1 and 7, isotope incorporation during the later stages of development was again not proportional to the concentration of individual BNP.

The results point to complex controls of BNP synthesis. If control is at the transcriptional level, individual histone repeats (histone variants) and even histone genes themselves may be transcribed at different times. It is tempting to suggest that the variations in time of maximum synthesis among the BNP are related to the developmental programme. Unfor-

tunately, we cannot check whether such variations normally occur during the cell cycle, since no means is as yet available to synchronize growing cultures.

Dramatic changes in protein patterns (detected by staining), particularly in BNP 8 and 9, were associated with the slug stage. These changes were transient, the original pattern reappearing at the preculmination stage, and seemed to be related to the developmental programme. When slugs were disaggregated the original pattern also reappeared and during re-aggregation did not change dramatically again. The slug-associated changes were apparently "once and for all". Bands 8 and 9 were synthesized between the finger and slug stages, but changes in pattern seemed too profound to be accounted for by synthesis alone. The reproducibility of the results speaks against some artifact (eg. proteases). The transient nature of the change suggests reversible protein modifications are involved.

Disaggregation Experiments

When developing *D. discoideum* cultures are disaggregated the cells are capable of recapitulating the same sequence of previous morphological changes and in a fraction of the original time (149, 150). The accumulation of enzymes (149, 150) and the synthesis of plasma membrane proteins (151) which correlate with specific developmental stages are repeated during redifferentiation. Irrespective of the amount of these enzymes present when cells are disaggregated, they make the full quantum of enzyme associated with differentiation. One explanation is that the disaggregated cells are unable to utilize stored transcripts so that reaggregation switches on again the programmed transcription of the enzyme mRNA which is rapidly translated.

Disaggregation experiments showed that, like slug DNA synthesis (65), the synthesis of eight BNP is a once and for all event. Hence, if cells were disaggregated at a stage when synthesis

of one of these proteins had commenced, its synthesis ceased and was not reinitiated during reaggregation. The synthesis of six BNP (numbers 1, 3, 5, 7, 8 and 12) was, however, invariably reinitiated in reaggregating cells.

One interpretation of these results would be that the eight BNP no longer synthesized following disaggregation are involved in some other once and for all event such as DNA synthesis. The synthesis of the other six BNP may be more closely allied to the modulation of gene activity associated with development. However, it must be remembered that, although their synthesis was subject to variable controls, no BNP (histone variants ?) appeared to be uniquely associated with a particular stage of differentiation.

Modifications of BNP

Histone modification may be involved in the temporary interruptions of histone-histone or DNA-histone interactions thought necessary if RNA polymerase is to traverse around the DNA of nucleosomes. Acetylation is a more likely candidate than phosphorylation since the latter occurs only to a small degree in non-dividing cells (152) and the non-core histone H1 is mainly involved.

a) Acetylation

Acetylation and deacetylation are rapid and occur on all nucleosomal histones (153, 154, 155). However, the steady-state level of mono-modification is rather high, amounting to 30-40% of the total histone (154). Extensive modification, ie. three or four acetate groups per histone, accounts for a relatively small fraction of the total histone and could be important in allowing gene transcription. The nucleosome structure of highly acetylated chromatin is altered to render these nucleosomes selectively sensitive to DNAase I (156-159). Active genes also possess a DNAase I-sensitive structure (160).

We examined BNP acetylation in two ways. Firstly, we compared the incorporation of acetate with that of arginine/lysine during differentiation. Acetate incorporation in the absence of synthesis was taken as evidence of acetylation. Six BNP were clearly acetylated and acetylation continued until the latest stages of differentiation that could be studied (culmination). Four of the six BNP which are resynthesized following disaggregation, and hence possibly involved in the developmental programme (see above), were also acetylated. Most of the other BNP appeared to be only very weakly acetylated.

Secondly, acetate incorporation in the presence of cycloheximide was examined. Unfortunately, the results were ambiguous, presumably because cycloheximide interferes with processes other than protein synthesis (161, 162).

b) Phosphorylation

Three histone fractions are extensively phosphorylated in dividing cells and H1 and H3 phosphorylation is thought to be important in chromatin condensation into metaphase chromosomes (13, 163, 164). Histone phosphorylation, mainly of H1, does occur in dividing cells but only to a small degree (152, 165-169). Furthermore, interphase histone phosphorylation and its subsequent turnover occur at relatively low rates (eg. $t_{1/2}$ for H1 phosphate hydrolysis is 4-5 hours) (170).

We found two BNP (bands 1/2 and 4/5, absolute identification being impossible) strongly phosphorylated in *D. discoideum* nuclei *in vitro*. The BNP equivalent to H1 (band 1/2) was dephosphorylated much slower than the other labelled proteins, suggesting either specific phosphatase exists or BNP 1/2 was shielded from phosphatase activity. Strong phosphorylation of BNP 10 occurred during mid-development.

Separation of the phosphorylated BNP on 2D gels showed, firstly, different degrees of phosphorylation of the same proteins

probably occur and, secondly, only a relatively small proportion of the individual proteins are phosphorylated.

Phosphorylation of BNP during differentiation was examined *in vitro* and *in vivo*. The *in vivo* labeling gave different results. This may be because the nuclear pool of labelled ATP remained low *in vivo* or, more likely, because isolation of nuclei leads to subtle changes in chromatin structure, exposing different proteins to protein kinases. Activation of protein kinases may also occur. Labeling of BNP *in vivo* and *in vitro* continued throughout most of development, decreasing in the final stages. Phosphorylation of BNP 1/2 was detected both *in vitro* and *in vivo* and continued relatively constant until the tip stage (in some experiments until slugs). If phosphorylation of these proteins is involved in chromatin condensation, it appears earlier than would be expected, the S-phase of second mitotic division reaching a maximum in slugs (65).

The sensitivity of kinase activity to changes in chromatin structure was indicated by the discovery that phosphorylation was greatly reduced when nuclei were incubated at pH 5. This activity could be restored by DNAase I treatment. DNAase II also partly overcame the inhibition and, if cAMP and particularly cGMP were present, strong phosphorylation was again detected. This suggests a cyclic nucleotide-sensitive protein kinase does occur in nuclei but loses its sensitivity when nuclei are isolated. Changes in chromatin structure appear to be involved. (It is relevant that calf thymus histones added to the nuclear homogenate were not phosphorylated).

In vitro phosphorylation of nuclei and nucleoli from stationary cells was compared. The major difference was the presence of a strongly phosphorylated protein (mol. wt. 17,000) in nucleoli. We did not include this protein among the 14 BNP although it could be weakly detected in the BNP

and chromatin fractions. It was also found in nucleosomes monomers and dimers. Kühn et al. (171) recently showed a nucleolar phospho-protein may specifically regulate ribosomal DNA functions in *Physarum*.

General

The results showed 14 BNP were present in nuclei and 12 of these in nucleosome dimers. These 12 BNP may include histone variants (eg. numbers 1 and 2), although their relationship to one another awaits amino-acid analysis. We do not know whether the 12 BNP are present in all nucleosomes and have not attempted to examine their relative concentration in nucleosomes. It would be useful if different nucleosome populations could be isolated (eg. with nucleases). The similarity between BNP from nucleoli and chromatin speaks against gene-specific associations of BNP.

BNP were synthesized during development, but not in a block as might be expected if synthesis were subject to a simple control. (The results do not warrant a discussion on transcriptional as against translational control of histone synthesis (172)). Although mitotic division does occur during differentiation, no clear temporal correlation was found between BNP synthesis or phosphorylation and DNA synthesis. The labeling experiments did not indicate that any unique BNP were synthesized during differentiation. If stage-specific histone variants are synthesized they must be present in very low concentrations. The unique pattern of BNP found in slugs was apparently due to protein modifications rather than synthesis of new BNP.

Six of the BNP, all found in nucleosome monomers, were resynthesized during the redifferentiation following disaggregation. In this they resemble other proteins known to be coded for by the developmental programme (151). It would be advantageous to compare synthesis of BNP during differentiation and the cell cycle. However, no means for adequately synchronizing vege-

tative cells has been found.

The number of proteins synthesized decreases throughout differentiation (173) and transcription probably also diminishes. The newly synthesized BNP may be involved in genome repression. BNP were increasingly difficult to isolate during differentiation, suggesting the strength of DNA: BNP association increases.

Acetylation of BNP occurred throughout differentiation. It will be interesting to see whether acetylated BNP are preferentially released from chromatin by nucleases which digest active genes (160). Developmentally controlled genes, required to monitor the sensitivity of active genes to nucleases, are now being cloned (J. Williams, personal communication).

The role of BNP phosphorylation in differentiation remains a mystery, no correlation with DNA synthesis having been found. Protein kinase(s) and phosphatase(s) were detected in nuclei and the importance of subtle changes in chromatin structure on kinase activity observed. We obtained first evidence of a cGMP-dependent protein kinase. Extracellular cAMP is known to induce the synthesis of developmentally regulated plasma membrane proteins (174,175) and cGMP is synthesized intracellularly in response to extracellular cAMP pulses (176, 177).

V. SUMMARY

Basic nuclear proteins (BNP) were isolated from nuclei of axenic and wild type strains of *Dictyostelium discoideum* and examined with SDS and acid-urea polyacrylamide gel electrophoresis.

Two dimensional gel electrophoresis was used to determine the relationship between BNP separated by the two systems. A number of minor components were present in the BNP and may represent secondarily modified proteins.

Chromatin, nucleoli, nuclear membranes and a "crude" nucleosomal fraction were isolated and their BNP content examined. Nucleosome monomers and dimers were isolated using gel electrophoresis. Fourteen BNP were extracted from nuclei and twelve of these were found in nucleosomes. Hence they are probably histones.

Nucleoli and non-nucleolar chromatin showed no marked differences in BNP content. However, nucleoli do possess a distinctive chromatin structure and the association between BNP and DNA was weaker than in non-nuclear chromatin.

During development the BNP became increasingly difficult to isolate from DNA. Fortunately, the fourteen BNP were not contaminated by other proteins after nuclear homogenates had been electrophoresed on SDS gels. Whole nuclei from different developmental stages could then be used to examine changes in BNP. Only small variations in the relative concentrations of BNP occurred during differentiation. The slug stage proved exceptional, where the reproducible but transient changes appeared to be due to BNP modifications rather than synthesis.

The synthesis of BNP during differentiation was examined by labelling with radioactive arginine and lysine or acetate. No new and stage-specific BNP were detected. The BNP were

all synthesized at some time during development. The synthesis of eight BNP ceased or was drastically reduced at the finger stage. Synthesis of the remaining BNP continued beyond the finger stage and four of them exhibited their strongest isotope incorporation during this period. No temporal correlation was found between BNP synthesis and the two periods of DNA synthesis (preaggregation and slug stages) when cell numbers increase by 15-20%

It is known that when developing aggregates are disaggregated and replated, development is recapitulated and developmentally regulated proteins resynthesized. Disaggregation experiments showed the synthesis of eight BNP was a once and for all event. If cells were disaggregated at a stage when synthesis of one of these proteins had commenced, its synthesis ceased and was not reinitiated during reaggregation. The synthesis of six BNP, all associated with nucleosome monomers, was invariably reinitiated in reaggregating cells.

The controls of BNP synthesis are apparently complex. Synthesis during the cell cycle could not be studied as no way is known to synchronize growing *Dictyostelium* cultures.

Six BNP were strongly acetylated and acetylation continued until the final stage of development.

Phosphorylation was examined both *in vivo* and *in vitro*. Protein kinase and phosphatase activities were detected in the nucleus. Dephosphorylation of the "H1-like" BNP was much slower than other phosphorylated BNP. Cyclic nucleotides and polyamines had no influence on *in vitro* phosphorylation. Two dimensional gel electrophoresis indicated various degrees of phosphorylation occurred and only a small proportion of the individual protein molecules were phosphorylated.

Changes in phosphorylation during development were examined. Very different labelling patterns were obtained with *in vitro* and *in vivo* phosphorylation. Changes in chromatin structure occurring during nuclei isolation may modify substrate availability or activate protein kinase(s). Both *in vitro* and *in vivo* experiments showed the "H1-like" BNP were phosphorylated during differentiation. However, no temporal correlation with DNA synthesis was observed. Preincubation of nuclei at pH 5 inhibited protein kinase activity. This inhibition was overcome by DNAase I treatment. DNAase II only partially overcame inhibition, but in the presence of cGMP (10^{-6} M) the protein kinase was again fully active.

ZUSAMMENFASSUNG

Aus den Kernen von axenischen und Wildtyp-Stämmen von *Dictyostelium discoideum* wurden Basische Kernproteine (BNP) isoliert und mit SDS und Harnstoff-Polyacrylamid-Gelelectrophorese untersucht. Mit Hilfe der zweidimensionalen Gelelectrophorese wurde überprüft, in welchem Verhältnis die mit den beiden Methoden getrennten BNP zueinander stehen. Eine Anzahl kleinerer Komponenten waren in den BNP vorhanden, bei welchen es sich wohl zum Teil um sekundär modifizierte Proteine handelt.

Chromatin, Nucleoli, Kernmembranen und eine "crude nucleosomal fraction" wurden isoliert und auf ihren BNP-Gehalt hin überprüft. Nucleosomen- Monomere und -Dimere wurden mittelst Gelelectrophorese isoliert. Vierzehn BNP wurden aus Kerne isoliert; zwölf davon wurden in Nucleosomen gefunden, wahrscheinlich sind dies die Histone.

Nucleoli und nicht- nucleoläres Chromatin zeigten nur einen geringen Unterschied in ihrem Gehalt an BNP. Allerdings haben Nucleoli eine deutlich verschiedene Chromatinstruktur, und die Bindung zwischen BNP und DNA ist schwächer als in nicht-nucleolären Chromatin.

Im Laufe der Entwicklung wurde es zunehmend schwieriger, die BNP von der DNA zu trennen. Glücklicherweise waren die vierzehn BNP auf den SDS- Gelen des Kernhomogenates nicht durch andere Proteine kontaminiert. Veränderungen in den BNP könnten somit an ganzen Kernen der entsprechenden Entwicklungsstadien untersucht werden. Während der Differenzierung traten nur geringfügige Änderungen in der relativen Konzentration der BNP auf. Eine Ausnahme bildet das "Slug-Stadium", wo reproduzierbare, aber vorübergehende Änderungen offensichtlich eher auf BNP-Modifikationen, als auf Synthese zurückzuführen sind.

Die Synthese der BNP während der Differenzierung wurde durch Markierung mit radioaktivem Arginin und Lysin oder Acetat

untersucht. Es wurden keine neuen, stadiumspezifischen BNP gefunden. Alle BNP wurden während der Entwicklung synthetisiert. Im "Finger-Stadium" wurde die Synthese von acht BNP eingestellt, oder zumindest drastisch reduziert. Hingegen wurde die Synthese der restlichen BNP über das "Finger-Stadium" hinaus fortgesetzt; vier davon zeigten in diesem Stadium stärksten Isotopeneinbau. Es wurde keine zeitliche Korrelation beobachtet zwischen BNP-Synthese und den zwei Perioden der DNA-Synthese (Präaggregation und "Slug-Stadium"), während der sich die Zellzahl um 15-20% erhöht.

Es ist bekannt, dass in Entwicklung begriffene Aggregate nach dem Disaggregieren die Differenzierung wieder aufnehmen, und dass die durch die Entwicklung regulierten Proteine resynthetisiert werden. Disaggregationsversuche haben gezeigt, dass die Synthese von acht BNP ein für alle Mal geschieht. Wurden die Zellen in einem Stadium disaggregiert, in dem die Synthese eines dieser acht Proteine angelaufen war, dann wurde die Synthese eingestellt und während der folgenden Re-Aggregation nicht wieder aufgenommen. Hingegen setzte die Synthese von sechs anderen BNP, welche alle mit Nucleosomen- Monomeren assoziiert sind, stets wieder ein in reaggregierenden Zellen.

Die Kontrollmechanismen der BNP-Synthese sind offensichtlich komplex. Die Synthese während des Zellzyklus konnte nicht studiert werden, weil bisher keine Methode zur Erzielung von Synchronkulturen von Dictyostelium gefunden wurde.

Sechs BNP wurden stark acetyliert; die Acetylierung dauerte bis zum Ende der Entwicklung an.

Die Phosphorylierung wurde in vivo und in vitro studiert. Proteinkinase- und -phosphatase- Aktivität wurden im Kern gefunden. Die Dephosphorylierung der "H1- artigen" BNP verlief wesentlich langsamer als jene der anderen phosphorylierten BNP. Cyclische Nucleotide und Polyamine hatten keinen Einfluss auf die in vitro Phosphorylierung. Zweidimensionale Gelelektrophorese lässt auf

verschieden starke Phosphorylierung schliessen; nur ein kleiner Anteil der einzelnen Proteinmoleküle wurde phosphoryliert.

Es wurden die Änderungen in der Phosphorylierung während der Entwicklung untersucht. Sehr unterschiedliche Markierungsmuster wurden durch in vitro und in vivo Phosphorylierung erzielt. Veränderungen in der Chromatinstruktur während der Isolierung der Kerne könnten möglicherweise die Substratverfügbarkeit modifizieren, oder aber Proteinkinasen aktivieren. Sowohl die in vitro-, als auch die in vivo- Experimente zeigen, dass die "H1-artige" BNP während der Differenzierung phosphoryliert wird. Trotzdem konnte keine zeitliche Korrelierung mit der DNA-Synthese beobachtet werden. Praeinkubation der Kerne bei pH 5 hemmte die Proteinkinase- Aktivität. Diese Hemmung wurde durch DNAase I- Behandlung aufgehoben. DNAase II vermochte die Hemmung nur teilweise zu beheben, aber in Gegenwart von cGMP (10^{-6} M) wurde die Proteinkinase wieder voll aktiv.

VI. REFERENCES

1. Bonner, J. (1965) The molecular biology of development, Oxford Univ. Press.
2. Bonner, J., Dahmus, M.E., Fambrough, D., Huang, R.C., Marushige, K. and Tuan, D.Y.H., (1968) Science 159, 47-56
3. Miescher, F. (1897) in: Die histochemischen und physiologischen Arbeiten von Friedrich Miescher, F.C.W. Vogel, Leipzig
4. Kossel, A. (1918) The Protamine and Histones. Longmaus, Green and Co., London.
5. Stein, G. and Stein, J. (1976) Bio Science 26, 488-498
6. Stedman, E. and Stedman, E. (1950) Nature 166, 780-781
7. De Lange, R.J., Fambrough, D.M., Smith, E.L. and Bonner J. (1969) J. Biol. Chem. 244, 5669-5679
8. De Lange, R.J. and Smith, E.L. (1975) CIBA Found. Symp. 28, 59-76
9. De Lange, R.J., Hooper J.A. and Smith, E.L. (1973) J. Biol. Chem. 248, 3262-3274
10. Hooper, J.A., Smith, E.L., Sommer, K.R. and Chalkley, R. (1973) J. Biol. Chem. 248, 3275-3279
11. Tatty, L., Smith, E.L. and Johnson, J. (1973) J. Biol. Chem. 248, 6834-6844
12. Felsenfeld, G. (1978) Nature 271, 115-122
13. Elgin, S.C.R. and Weintraub, H. (1975) Ann. Rev. Biochem. 44, 725-774
14. Kornberg, R.D. (1977) Ann. Rev. Biochem. 46, 931-954
15. Pardon, J.F., Wilkins, M.H.F. and Richards, B.M. (1967) Nature 215, 508-509
16. Richards, B.M. and Pardon, J.F. (1970) Exp. Cell Res. 62, 184-196
17. Pardon, J.F. and Wilkins, M.H.F. (1972) J. Mol. Biol. 68, 115-124
18. Olins, A.L. and Olins, D.E. (1974) Science 183, 330-332
19. Oudet, P., Gross-Bellard, M. and Chambon, P. (1975) Cell 4, 281-300
20. Woodcock, C.L.F., Safer, J.P. and Stauchfield, J.E. (1976)

- Exp. Cell Res. 97, 101-110
21. Woodcock, C.L.F. (1973) J. Cell Biol. 59, 368 a
 22. Clark, R. and Felsenfeld, G. (1971) Nature 229, 101-106
 23. Thomas, J.O. and Kornberg, R.D. (1975) Proc. Natl. Acad. Sci. USA 72, 2626-2630
 24. Camerini-Otero, R.D., Sollner-Webb, B. and Felsenfeld, G. (1976) Cell 8, 337-347
 25. Sollner-Webb, B. and Felsenfeld, G. (1975) Biochemistry 14, 2915-2920
 26. Axel, R. (1975) Biochemistry 14, 2921-2925
 27. Shaw, B.R., Herman, T.M. Kovacic, R.T., Beandreau, G.S. and Van Holde, K.E. (1976) Proc. Natl. Acad. Sci. USA 73, 505-509
 28. Mirzabekov, A.D., Shick, V.V., Belyavsky, A.V. and Bavykin, S.G. (1978) Proc. Natl. Acad. Sci. USA 75, 4184-4188
 29. Finch, J.T., Lutter, L.C., Rhodes, D., Brown, R.S., Rushton B., Lewitt, M. and Klug, A. (1977) Nature 269, 29-36
 30. Van Holde, K.E., Sahasrabuddhe, C.G. and Shaw, B. (1974) Nucl. Acid. Res. 1, 1579-1586
 31. Baldwin, J.P., Boseley, P.G., Bradbury, M. and Ibel, K. (1975) Nature 253, 245-253
 32. Kornberg, R.D. (1974) Science 184, 868-871
 33. Hyde, J.E. and Walker, I.O. (1975) Nucl. Acid Res. 2, 405-421 and 1275-1289
 34. Varshavsky, A.J. and Georgiev, G.P. (1975) Mol. Biol. Rep. 2, 255-262
 35. Li, H.J. (1975) Nucl. Acid Res. 2, 1275-1289
 36. Weintraub, H., Worcel, A. and Alberts, B. (1976) Cell 9, 409-417
 37. Ord, M.G. and Stocken, L.A. (1966) Biochem. J. 98, 888-897
 38. Kleinsmith, L.J., Allfrey, V.G. and Mirsky, A.E. (1966) Proc. Natl. Acad. Sci. USA 55, 1182-1189
 39. Gershey, E.L., Vidali, G. and Allfrey, V.G. (1968) J. Biol. Chem. 243, 5018-5022
 40. De Lange, R.J., Smith, E.L., Fambrough, D.M. and Bonner, J. (1968) Proc. Natl. Acad. Sci. USA 61, 1145-1146
 41. Murray, K. (1964) Biochemistry 30, 10-15

42. Kim, S. and Paik, W.K. (1965) *J. Biol. Chem.* 240, 4629-4634
43. Sugimura, T. (1973) in *Prog. Nucleic Acid Res. Mol. Biol.* 13, (Davidson, J.N. and Cohn, W.E., eds) pp 127-151, Academic Press NY.
44. Sung, M.T. and Dixon, G.H. (1970) *Proc. Natl. Acad. Sci. USA* 67, 1616-1623
45. Dixon, G.H., Candido, E.P.M., Honda, B.M., Louie, A.J., MacLeod, A.R. and Sung, M.T. (1975) *Ciba Found. Symp.* 28 229-250
46. Raper, K.B. (1940) *J. Elisha Mitchell Sci. Soc.* 58, 241-282
47. Firtel, R.A. (1972) *J. Mol. Biol.* 66, 363-377
48. Newell, P.C. (1971) in *Essays in Biochemistry* 7, (Campbell, P.N. and Dickens, F., eds) pp 87-126, Academic Press, London
49. Ashworth, J.M. and Walts, D.J. (1970) *Biochem. J.* 119, 175-182
50. Sussman, R. and Raymer, E.P. (1971) *Arch. Biochem. Biophys.* 144, 127-137
51. Firtel, R.A. and Bonner, J. (1972) *J. Mol. Biol.* 66, 339-361
52. Katz, E.R. (1978) *Bio Science* 28, 692-697
53. Firtel, R.A., Johnson, P., Wright, C.K. Kindle, K.L. and Huxley, M.P. (1978) unpublished, citated in Firtel, R.A. and Jacobson, A. (1977) *Int. Rev. Biochem.* 15: 377-429
54. Alton, T.H. and Lodish, H.F. (1977) *Dev. Biol.* 60, 180-206
55. Loomis, W.F. Dimond, R., Free, S. and White, S. (1977) in *Eukaryotic microbes as model developmental system* (O'Day, D. and Hongen, P. eds) pp 177-194, Marcel Dekker, New York
56. Wilson, C.M. and Ross, I.K. (1957) *Am. J. Bot.* 44, 345-350
57. Coukell, M.B. and Walker, I.O. (1973) *Cell Diff.* 2, 87-95
58. Osborn, P.J. and Ashworth, J.M. (1975) *Cell Diff.* 4, 237-241
59. Charlesworth, M.C. and Parish, R.W. (1975) *Eur. J. Biochem.* 54, 307-316
60. Charlesworth, M.C. and Parish, R.W. (1977) *Eur. J. Biochem.* 75, 241-250
61. Parish, R.W., Stalder, J. and Schmidlin, S. (1977) *FEBS letters* 84, 63-66
62. Bakke, A.C., Wu, J.R. and Bonner, J. (1978) *Proc. Natl.*

- Acad. Sci. USA, 75, 705-709
63. Raper, K.B. (1941) *Growth* 5, 41-76
 64. Sussman, R.R. and Sussman, M. (1960) *J. Gen. Microbiol.* 23, 287-293
 65. Zada-Hames, I.M. and Ashworth, J.M. (1978) *Develop. Biology* 63, 307-320
 66. Konijn, T.M. (1972) *Acta Protozoologica* 11, 137-143
 67. Konijn, T.M., Van de Meene, J.G., Bonner, J.T. and Barkley, D.S. (1967) *Proc. Natl. Acad. Sci. USA* 58, 1152-1154
 68. Cocucci, S.M. and Sussman, M. (1970) *J. Cell Biol.* 45, 399-407
 69. Raper, K.B. (1951) *Quart. Rev. Biol.* 26, 169-190
 70. Sussman, M. (1961) *J. Gen. Microbiol.* 25, 375-378
 71. Bonner, J.T. (1967) *The Cellular Slime Moulds*, 2nd edn., Princeton University Press, Princeton
 72. Newell, P.C. Telser, A. and Sussman, M. (1969) *J. Bact.* 100, 763-768
 73. Firtel, R.A., Jacobson, A. and Lodish, H.E. (1972) *Nat. New. Biol.* 239, 225-228
 74. Comings, D.E. and Harris, D.C. (1976) *J. Cell Biol.* 70, 440-452
 75. Nelson, D.A., Beltz, W.R. and Rill, R.L. (1977) *Proc. Natl. Acad. Sci. USA* 74, 1343-1347
 76. Wang, S., Chin, J.F., Klyszejko-Stefanowicz, L., Fujitani, H., Hnilica, L.S. and Ansevin, A.T. (1976) *J. Biol. Chem.* 251, 1471-1475
 77. Panyim, S. and Chalkley, R. (1969) *Arch. Biochem. Biophys.*, 130, 337-346
 78. Reisner, A. H., Nemes, P. and Bucholty, C. (1975) *Anal. Biochem.* 64, 509-516
 79. Hurley, C.K. (1977) *Anal. Biochem.* 80, 624-626
 80. Laemmli, U.K. (1970) *Nature (London)* 227, 680-685
 81. Unger-Ullmann, C. and Modak, S.P. (1978) in *Abstracts of the 10th Annual Meeting of the Union of Swiss Societies of Experimental Biology*
 82. Compton, J.L., Bellard, M. and Chambon, P. (1976) *Proc. Natl. Acad. Sci. USA* 73, 4382-6386

83. Staron, K., Jerzmanowski, A., Tyniec, B., Urbanska, A. and Toczko, K. (1977) *Biochem. Biophys. Acta* 475, 131-138
84. Woodcock, C.L.F., Sweetman, H.E. and Frado, L.L. (1976) *Exp. Cell Res.* 97, 111-119
85. Todd, R.D. and Garrard, W.T. (1977) *J. Biol. Chem.* 252, 4729-4738
86. Varshavsky, A.J., Bakayev, V.V. and Georgiev, G.P. (1976) *Nucl. Acid Res.* 3, 477-492
87. Spurr, A.R. (1969) *J. Ultrastructure Res.* 26, 31-43
88. Sampson, J. (1977) *Cell* 11, 173-180
89. Farron-Furstenthal, F. and Lightholder, J. R. (1978) *Biochem. Biophys. Res. Commun.* 83, 94-100.
90. Keely, S.L., Corbin, J.D., Park, C.R. (1975) *Proc. Natl. Acad. Sci. USA* 72, 1501-1504
91. Böhm, J., Keil, G. and Knippers, R. (1977) *Eur. J. Biochem.* 78, 251-266
92. Sherod, D., Johnson, G. and Chalkley, R. (1970) *Biochemistry* 9, 4611-4615
93. Bonner, W.M. and Laskey, R.A. (1974) *Eur. J. Biochem.* 46, 83-88
94. Chapleo-Charlesworth, M. (1976) *The Isolation of Nuclei and Basic Nucleoproteins from the Cellular Slime Mould Dictyostelium discoideum*, Inaugural Dissertation, University Zurich
95. Späth, P.J. and Koblet, H. (1979) *Anal. Biochem.* 93, 275-285
96. Judd, W. (1979) *Anal. Biochem.* 93, 373-379
97. Drescher, D.G. and Lee, K.S. (1978) *Anal. Biochem.* 84, 559-569
98. Parish, R.W., Schmidlin, S. and Parish, C.R. (1978) *FEBS Letters* 95, 366-370
99. Pederson, T. (1977) *Biochemistry* 16, 2771-2777
100. Firtel, R.A. and Pederson, T. (1975) *Proc. Natl. Acad. Sci. USA* 72, 301-305
101. Rizzo, P.J. and Noodén, L.D. (1974) *Biochem. Biophys. Acta* 349 402-414
102. Jockusch, B.M. and Walker, I.O. (1974) *Eur. J. Biochem* 48, 417-425

103. Widmer, R., Fuhrer, S., Parish, R.W. (1979), FEBS Letters 106, 363-369
104. Fellenberg, G. (1974) Chromosomale Proteine, Eugen Ullmer Verlag, Stuttgart
105. Liew, C.C. and Chan, P.K. (1976) Proc. Natl. Acad. Sci. USA 73, 3458-3462
106. Johnson, E.M., Matthews H.R., Lothstein, L., Bradbury, E.M. and Allfrey, V.G. (1978) Proc. Natl. Acad. Sci. USA 75, 1116-1120
107. Olins, A.L., Carlson, D.R., Wright, E.B. and Olins, D.E. (1976) Nucl. Acid Res. 3, 3271-3291
108. Johns, E.W., Goodwin, G.H., Walker, J.M. and Sanders, C. (1975) Ciba Found. Symp. 28, 95-108
109. Goodwin, G.H., Nicolas, R.H. and Johns, E.W. (1975) Biochim. Biophys. Acta 405, 280-291
110. Spiker, S., Mardian, J.K.W. and Isenberg, I. (1978) Biochim. Biophys. Res. Comm. 82, 129-135
111. Goodwin, G.H. and Johns, E.W. (1978) Biochim. Biophys. Acta 519, 279-284
112. Goodwin, G.H., Woodhead, L. and Johns, E.W. (1977) FEBS Letters 73, 85-88
113. Yu, S.S., Li, H.J., Goodwin, G.H. and Johns, E.W. (1977) Eur. J. Biochem, 78, 497-502
114. Javaherian, K., Liu, L.F. and Wang, J.C. (1978) Science 199, 1345-1346
115. Johns, E.W. (1964), Biochem. J. 92, 55-59
116. Sung, M.T., Harford, J., Bundman, M. and Vidalakas, G. (1977) Biochemistry 16, 279-285
117. Bakayev, V.V. Bakayeva, T.G., Schmatchenko, V.V. and Georgiev, G.P. (1978), Eur. J. Biochem. 91, 291-301
118. Birnstiel, M., Telford, J., Weinberg, E. and Stafford, D. (1974) Proc. Natl. Acad. Sci. USA 71, 2900-2904
119. Clarkson, S., Smith, H., Schaffner, W., Gross, K.W. and Birnstiel, M. (1976) Nucl. Acids Res. 3, 2617-2632
120. Kedes, L.H., Chang, A.C., Housman, D. and Cohen, S. (1975) Nature 255, 533-538
121. Schaffner, W., Gross, K., Telford, J. and Birnstiel, M.

- (1976) Cell 8, 471-478
122. Gross, K., Schaffner, W., Telford, J. and Birnstiel, M.
(1976) Cell 8, 479-484
 123. Cohn, R.H., Lowry, J.C. and Kedes L.H. (1976) Cell 9,
147-161
 124. Schaffner, W., Kunz, G., Daetwyler, H., Telford, J.
Smith, O.H. and Birnstiel, M.L. (1978) Cell 14, 655-671
 125. Kinkade, J.M., Cole, R.D. (1966) J. Biol. Chem. 241,
5790-5797
 126. Cohen, L., Newrock, K. and Zweidler, A. (1975) Science
190, 994-997
 127. Senger, D.R., Arceci, R.J. and Gross, P.R. (1978) Dev.
Biol. 65, 416-425
 128. Strickland, M., Strickland, N.W., Brandt, W.F. and von
Holt, C. (1977) Eur. J. Biochem. 77, 263-275
 129. Strickland, M., Strickland, N.W., Brandt, W.F. and von
Holt, C. (1977) Eur. J. Biochem. 77, 277-286
 130. Vidali, G., Boffa, L.C., Allfrey, V.G. (1977) Cell 12,
409-415
 131. Mathew, C.G.P., Goodwin, G.H. and Johns, E.W. (1979)
Nucl. Acids Res. 6, 167-179
 132. Weisbrod, S. and Weintraub, H. (1979) Proc. Natl. Acad.
Sci. USA 76, 630-634
 133. Prescott, D.M. (1966) J. Cell Biol. 31, 1-9
 134. Robbins, F. and Borun, T.W. (1967) Proc. Natl. Acad. Sci.
USA 57, 409-416
 135. Sadgopal, A. and Bonner, J. (1969) Biochim. Biophys. Acta
186, 349-357
 136. Takai, S., Borun, T.W., Muchmore, J. and Lieberman, I.
(1968) Nature 219, 860-861
 137. Gallwitz, D. (1975) Nature 258, 247-248
 138. Borun, T.W., Gabrielli, F., Ajiro, K., Zweidler, A. and
Baglioni, C. (1975) Cell 4, 59-67
 139. Gurly, L.R., Walters, R.A. and Tobey, R.A. (1972) Arch.
Biochem. Biophys. 148, 633-641
 140. Appels, R. and Wells, J.R.E. (1972) J. Mol. Biol. 70,
425-434.

141. Adamson, E.D. and Woodland, H.R. (1974) *J. Mol. Biol.* 88, 263-285
142. Weintraub, H. (1972) *Nature* 240, 449-453
143. Tsanev, R. and Russev, G. (1974) *Eur. J. Biochem.* 43, 257-263
144. Jackson, V., Granner, D.K. and Chalkley, R. (1975) *Proc. Natl. Acad. Sci. USA* 72, 4440-4444
145. Jackson, V., Granner, D.K. Chalkley, R. (1976) *Proc. Natl. Acad. Sci. USA* 73, 2266-2269
146. Grellet, F., Delseny, M., Guitton, Y. (1977) *Nature* 267, 724-726
147. Voordouw, G. and Eisenberger, H. (1978) *Nature* 273, 446-448.
148. Neelin, J.M., Mazen, A. and Champagne, M. (1976) *FEBS Letters* 65, 309-314
149. Newell, P.C., Longlands, M., Sussman, M. (1971) *J. Mol. Biol.* 58, 541-554
150. Newell, P.C., Franke, J. and Sussman, M. (1972) *J. Mol. Biol.* 63, 373-382
151. Parish, R.W., Schmidlin, S. (1979) *FEBS Letters* 99, 270-273
152. Gurley, L.R., Walter, R.A. and Tobey, R.A. (1974) *J. Cell Biol.* 60, 356-364
153. Ruiz-Carrillo, A., Wangh, L.J. and Allfrey, V.G. (1975) *Science* 190, 117-128
154. Jackson, V., Shires, A., Chalkley, R. and Granner, D. (1975) *J. Biol. Chem.* 250, 4856-4863
155. Jackson, V., Shires, A., Tamphaichtr, N. and Chalkley, R. (1976) *J. Mol. Biol.* 104, 471-483
156. Simpson, R.T. (1978) *Cell* 13, 691-699
157. Vidali, G., Boffa, L.C., Bradbury, E.M. and Allfrey, V.G. (1978) *Proc. Natl. Acad. Sci. USA* 75, 2239-2243
158. Sealy, L. and Chalkley, R. (1978) *Nucl. Acids Res.* 5, 1863-1876
159. Levy-Wilson, B. Watson, D.C. and Dixon, G.H. (1979) *Nucl. Acids Res.* 6, 259-274

160. Weintraub, H. and Groudine, M. (1976) *Science* 193, 848-856
161. Mc Mahon, D. (1975) *Plant Physiol.* 55, 815-821
162. Parish, R.W. (1976) *Biochim. Biophys. Acta* 444, 802-809
163. Bradbury, E.M. (1978) *Phil. Trans R. Soc. London* 13, 283, 291-293
164. Gurley, L.R., D'Anna, J.A., Barham, S.S., Deaven, L.L., Tobey, R.A. (1978) *Eur. J. Biochem.* 84, 1-15
165. Lake, R.S. and Salzman, N.P. (1972) *Biochemistry* 11, 4817-4826
166. Lake, R.S. (1973) *J. Cell Biol.* 58, 317-331
167. Balhorn, R., Bordwell, J., Seller, L. Granner, D. and Chalkley, R. (1972) *Biochem. Biophys. Res. Comm.* 46, 1326-1333
168. Balhorn, R., Chalkley, R. and Granner, D. (1972) *Biochemistry* 11, 1094-1097
169. Marks, D., Paik, W.K. and Borun, T.W. (1973) *J. Biol. Chem.* 248, 5660-5667
170. Balhorn, R., Jackson, V., Granner, D. and Chalkley, R. (1975) *Biochemistry* 14, 2504-2511
171. Kühn, G.D., Affolter, H.U., Atmar, N.J., Seebeck, T., Gubler, V., Braun, R. (1979) *Proc. Natl. Acad. Sci. USA* 76, 2541-2545
172. Melli, M., Spinelli, G. and Wyssling, H. (1977) *Cell* 11, 651-661
173. Parish, R.W. and Schmidlin, S. (1979) *FEBS Letters* 98, 251-256
174. Parish, R.W., Schmidlin, S. and Weibel M. (1978) *FEBS Letters* 96, 283-286
175. Parish, R.W. (1979) *Biochim. Biophys. Acta* 553, 179-182
176. Wurster, B., Schubiger, K., Wick, U. and Gerish, G. (1977) *FEBS Letters* 76, 141-144
177. Mato, J.M., Krens, F.A., van Haastert, P.J.M. and Konijn, T.M. (1977) *Proc. Natl. Acad. Sci. USA* 74, 2348-2351

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